STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE POLLED INTERVAL ON BOVINE CHROMOSOME 1

A Dissertation

by

KRIS RAKOWITZ WUNDERLICH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Genetics

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Approved by:

Chair of Committee,	Clare A. Gill
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	Thomas E. Spencer
Chair of Genetics Faculty,	James R. Wild

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ABSTRACT

Structural and Functional Characterization of the Polled Interval on Bovine Chromosome 1. (May 2008)

Kris Rakowitz Wunderlich, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Clare A. Gill

The horned condition in cattle is believed to be the wild type with morphogenesis primarily occurring after birth. The polled condition has existed since domestication and has been selected for its economic importance. The polled locus has previously been mapped by genetic linkage analysis to the proximal region of bovine chromosome 1. In order to help us eventually identify the causative mutation, the objective of the study was to structurally and functionally characterize the polled interval from *IFNAR1* to *SOD1* on BTA1. Our hypothesis was that the polled locus is a tissue specific transcription factor that is expressed in the developing horn buds and acts directly or indirectly upon SOX9.

A 2.5 Mb BAC contig and STS content map of the polled interval was constructed. Three candidate genes encoding transcription factors were identified within this region but only *C21orf66* was expressed in the horn buds from 1 d old *Bos indicus* influenced calves. The C21orf66 gene has 18 exons, spans 30,976 bp of genomic DNA, and 144 SNP were identified. No single SNP discovered in *C21orf66* can be attributed as the causative mutation.

None of the genes from the polled interval were differentially expressed in skin and horn from 1 d old *Bos indicus* influenced calves. However, there were significant differences in the levels of expression of *RUNX2*, *SOX9*, *BMP4*, *PRKCA*, and *FOXL2* in these samples. Expression of *RUNX2* was localized to the osteoblasts, and both *RUNX2* and *SOX9* were expressed in sebaceous glands of the horn at 1 d of age. Histological examination of horns and scurs from newborn, 5 to 6 mo, and ~1.5 yr old *Bos indicus* influenced cattle suggest that horns form through intramembranous ossification.

Based on the data presented herein, we propose that the polled locus is upstream of RUNX2 and SOX9 in the osteogenic pathway, and could have its primary effect on the differentiation of mesenchymal condensations. The genes *IL10RB*, *SFRS15*, *C21orf66*, *OLIG1*, *OLIG2* and *HUNK* remain candidates for the polled locus and warrant further investigation.

DEDICATION

I dedicate this to everyone who has supported and cheered me on during this process. To Pete, for listening, understanding, and standing beside me. I couldn't have come this far without your love and support. To my parents, for all of your help throughout my many years of school. Your pride in me helped me to stick to my goals.

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I also want to express sincere gratitude to Colette Abbey for teaching me countless lab techniques and helping with my research. My time in the lab has been much more enjoyable because of her, and I am happy to have made such a great friend. I also want to acknowledge all of my fellow graduate students and the student workers who have befriended me and helped out through the years. It has been great knowing all of you and I hope to stay in touch.

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To my loving husband, Pete, for your patience and support. I could not have maintained sanity, enthusiasm, or happiness without you. Finally, thanks to my family for their encouragement and helping to support my education. Your sacrifices did not go unnoticed.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Bone Development

Osteogenesis is the process where mesenchymal tissue is transformed into bone tissue (Gilbert, 1997). The bone structures are formed at specific times and sites by either intramembranous or endochondral ossification (Ueta et al., 2001). It is currently unclear whether the bony core of bovine horns is intramembranous or endochondral in nature.

Intramembranous bones are classified into 3 categories: the sesamoid bones, which form in tendons as a result of mechanical stress; the periosteal bones, which form from connective tissue and add to the thickness of long bones; and the dermal bones, which form within the dermis of the skin (Abzhanov et al., 2007). Intramembranous bones derive from the direct differentiation of mesenchymal cells into osteoblasts (Figure 1.1), which occurs in several craniofacial bones and the lateral part of clavicles (Nakashima and de Crombugghe, 2003). Osteoblasts are bone-precursor cells that secrete osteoid, a collagen-proteoglycan prebone matrix, which begins to calcify (Gilbert, 1997). These osteoblasts either become trapped within this calcified matrix and become osteocytes (bone cells), or they separate from the region of calcification as they secrete more osteoid, increasing the size of the bony spicule. These bony spicules fuse

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This dissertation follows the style of Journal of Animal Science.

with each other and become trabeculae. The periosteum is then formed around the trabeculae by differentiating mesenchymal cells, and bone growth continues at the surface of trabeculae (Gilbert, 1997). Intramembranous ossification is not characterized nearly as well as endochondral ossification, but a number of the same genes are common to both processes (Abzhanov et al., 2007).

Most vertebrate bones, especially the long bones, develop by endochondral ossification (Figure 1.2) where cartilage is formed from mesenchymal cells and subsequently replaced by bone (Mackie et al., 2007). According to Gilbert (1997), endochondral ossification can be divided into 5 stages: 1) mesenchymal cells are committed to become cartilage cells; 2) committed mesenchyme cells condense into compact nodules and differentiate into chondrocytes; 3) chondrocytes proliferate rapidly to form the model for the bone secreting a cartilage specific extracellular matrix; 4) chondrocytes stop dividing and become hypertrophic chondrocytes; and 5) the cartilage model is invaded by blood vessels and osteoclasts causing hypertrophic chondrocytes to undergo apoptosis. While osteoclasts degrade the cartilage matrix, osteoblasts produce a bone-specific matrix using the degraded cartilage matrix as a scaffold (de Crombrugghe et al., 2001). Both positive and negative signaling kinases and transcription factors, such as SRY-box 9 (**SOX9**) and runt-related transcription factor 2 (**RUNX2**), and the interactions among them determine whether the differentiated chondrocytes remain within cartilage elements or undergo hypertrophic maturation prior to ossification (Goldring et al., 2006).

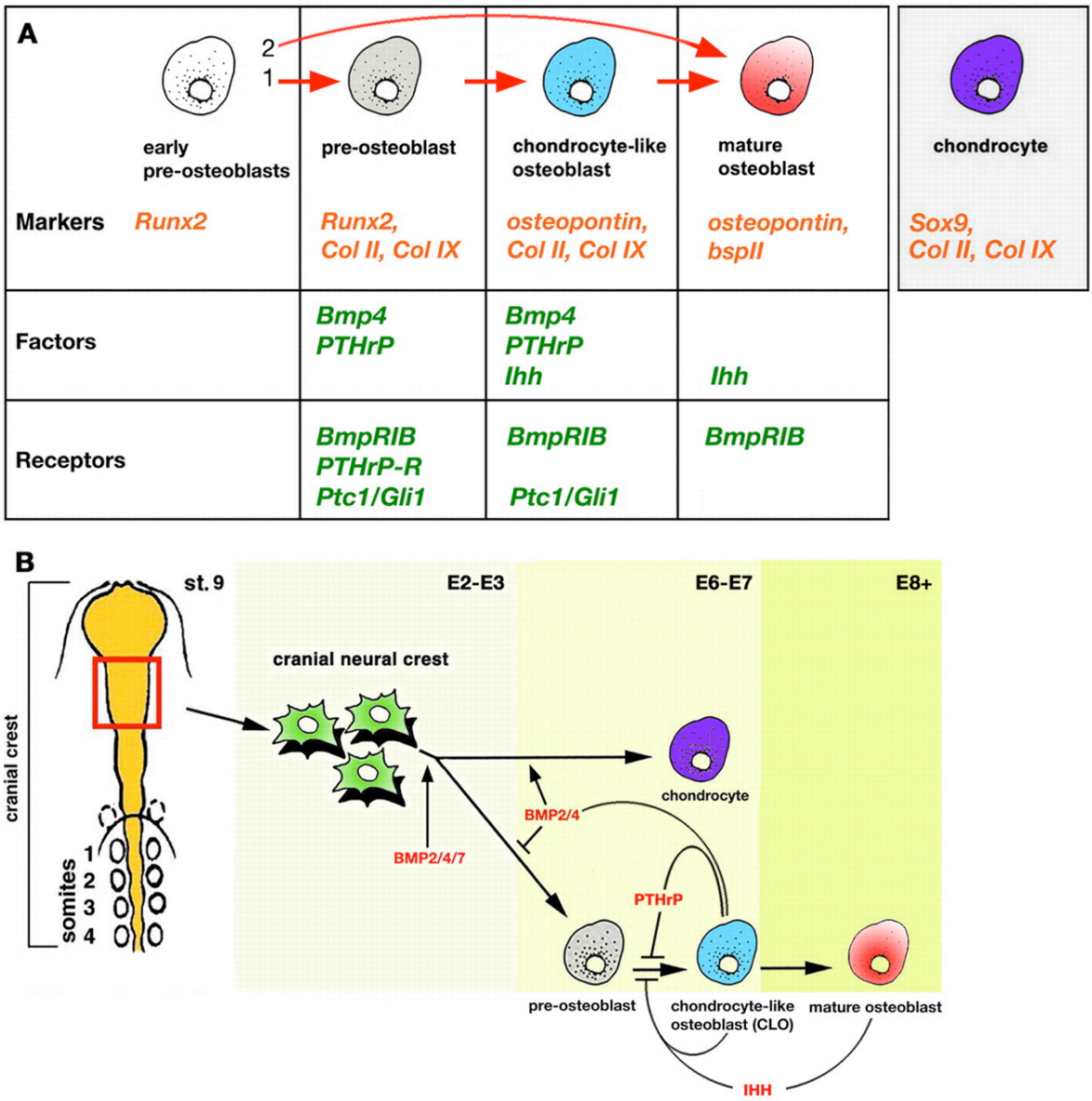

Figure 1.1. Regulation of osteoblastic differentiation in cranial dermal bone. (A) Four major cell types can be distinguished by the expression patterns of skeletogenic markers, factors and receptors. Early pre-osteoblasts may differentiate into mature osteoblasts via a chondrocyte-like osteoblast intermediate, or some cells may differentiate directly into mature osteoblasts. (B) During craniofacial development in mice, mesencephalic cranial neural crest cells migrate to populate mesenchyme of the future face and skull. Cells of the early cranial skeletogenic condensations depend on BMP2/4/7 activities to form preosteoblastic progenitors, whereas high levels of BMP2 and BMP4 induce a chondrogenic fate. Differentiation into chondrocyte-like osteoblasts is regulated by both IHH and PTHrP activities. (Abzhanov et al., 2007)

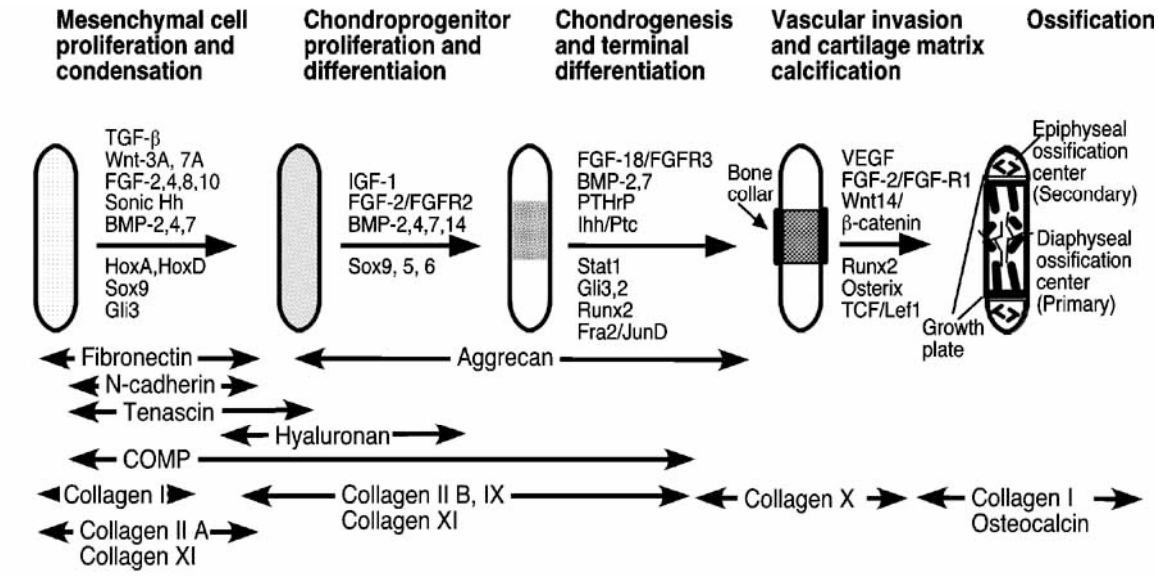

Figure 1.2. Sequence of events of chondrogenesis during the development of long bones. The different stages are represented schematically, showing the temporal patterns of growth and differentiation factors (above the arrows) and the transcription factors involved below the arrows. The extracellular matrix proteins that distinguish the different stages are indicated below. (Goldring et al., 2006)

Horns and Their Inheritance

As cited in Gadow (1902), Sandifort (1829) stated that the bony core of the bovine horn is a compound structure composed of a frontal outgrowth or pedicle, a superimposed ossification in a cartilaginous matrix (the os cornu), and a frontal sinus that will extend up into the pedicle and os cornu. This early description suggests that the bony horn core is endochondral in nature, but there is little histological data available (Fambach, 1901; Gadow, 1902). Dove (1935), similarly reported that the horn core is the result of a separate center of ossification originating in tissues above the periosteum that subsequently fuses to the frontal bone thereafter appearing as a simple exostosis or bony growth, whereby the os cornu has the power to draw up the frontal bone as support (Figure 1.3). Horns are covered by a tough shell of modified epithelium that grows outward from the skin at the base of the horn (Georges et al., 1993).

As described by Long and Gregory (1978), the horned phenotype is due to a homozygous recessive gene (pp). The polled phenotype is due to a dominant mutation (P) (Table 1.1). A separate gene affects the growth of scurs, which are generally small, horn-like growths above the frontal bone in the same location as where horns normally

grow (Asai et al., 2004). However, the size of scurs can vary from small and scab-like to large and horn-like (Asai et al., 2004). This can lead to errors in classification such as: scurred animals mislabeled as smooth polled, heavy scurred animals mislabeled as horned, and horned animals mislabeled as scurred. Scurs (Sc) appear to be expressed as a sex-influenced trait with incomplete penetrance.

A third locus affecting horn growth has also been proposed (Buchanan Smith, 1927). This locus later became known as the African horns gene (Ha), and it is believed to be epistatic to polled (P) in males and not epistatic to polled (P) in females and, thus, inherited in the same sex-limited fashion as scurs (White and Ibsen, 1936). The gene has been reported to be predominant in breeds native to Africa, but is present in other breeds (White and Ibsen, 1927; Williams and Williams, 1952). White and Ibsen (1936) suggested that scurs and African horns may be allelic.

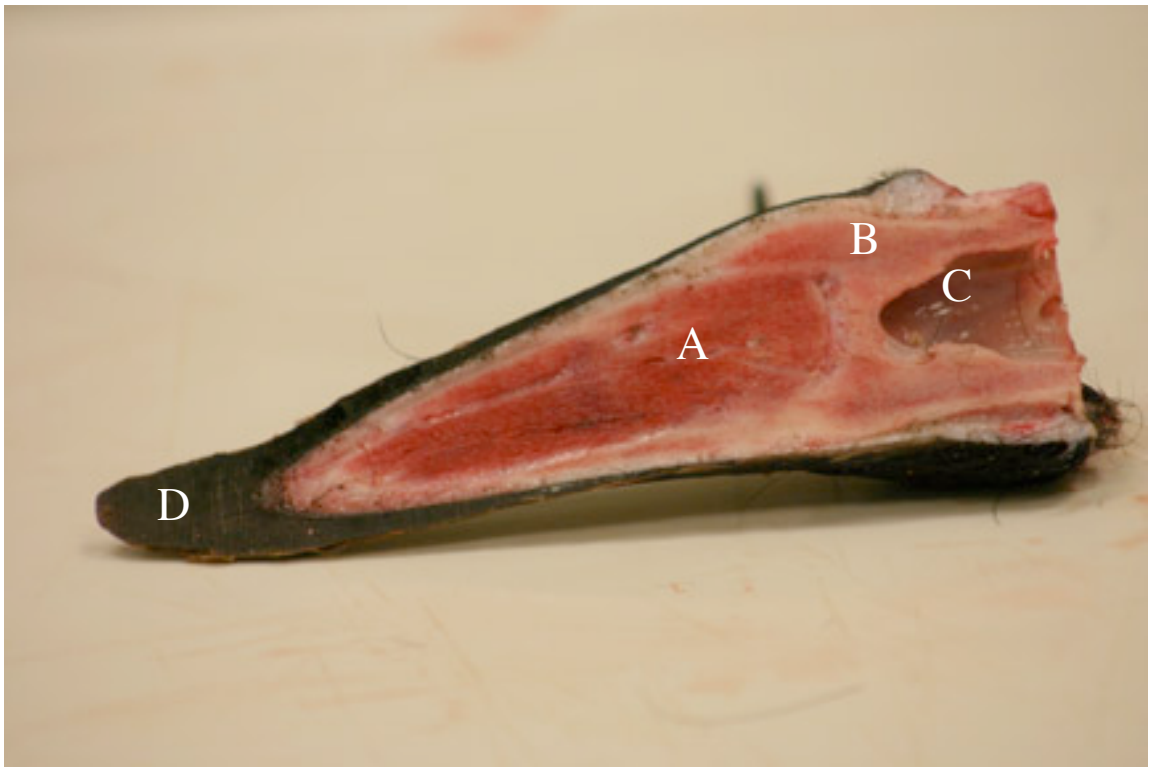

Figure 1.3. Dissection of a horn. This dissection demonstrates a separate center of ossification (A) that will subsequently fuse to the frontal bone (B) and the sinus cavity (C) pervades up into the horn. The horn is covered by a keratin sheath (D). (Photo courtesy of Dr. Clare Gill, Texas A&M University)

Table 1.1. Assumed inheritance model for the polled, horned, and scurred condition[1]

Genotype	**Males**	**Females**
PP ScSc	Scurred	Scurred
PP Scsc	Polled, nonscurred	Polled, nonscurred
PP scsc	Polled, nonscurred	Polled, nonscurred
Pp ScSc	Scurred	Scurred
Pp Scsc	Scurred	Polled, nonscurred
Pp scsc	Polled, nonscurred	Polled, nonscurred
pp ScSc	Horned	Horned
pp Scsc	Horned	Horned
pp scsc	Horned	Horned

[1]Long and Gregory, 1978

Economic Importance

Horns pose a threat to both animal handlers and other cattle. It has been estimated that horns cost beef producers $22 million in losses due to carcass bruising each year. Producers and packers ranked bruising as one of their top 10 concerns for the fed steer and heifer industry in the National Beef Quality Audit-2000 (McKenna et al., 2002). Bruising was also the number 2 'quality challenge' of the market (cull) cow and bull beef industry (Roeber et al., 2000). According to the National Beef Quality Audit-2005, there has been very little change in the percentage of animals with horns and levels of bruising in comparison to previous audits (Smith et al., 2006).

Dehorning of cattle by surgical means has become a temporary solution to the problem, but at the expense of loss of production (Goonewardene and Hand, 1991), and can cause animal welfare concerns resulting in negative effects on the beef market. Due to the management problems associated with horned cattle and the inconvenience and cost of dehorning, selective breeding for polled cattle may be a welfare-friendly method of eliminating the need to dehorn cattle (Goonewardene et al., 1999). However, the inability to differentiate between homozygous and some heterozygous polled animals poses a challenge in the selective breeding of polled cattle due to the recessive inheritance of the horned allele. Failure to detect carriers of the recessive horned allele maintains the horned allele, and horns, in the population. This challenge could be minimized through the use of genetic testing, which is most accurate when the causative mutation or mutations are known.

Genetic Mapping

Initially, Bricker and Church (1991) determined that the polled locus was located on either BTA1 or BTA29 based upon the cosegregation of the polled locus and a 1:29 Robertsonian translocation in Charolais cattle. Later, Georges et al. (1993) localized the polled locus to the centromeric end of BTA1 by microsatellite mapping. Brenneman et al. (1996) refined the location of polled to a region proximal to the centromere and 4.9 cM from the genetic marker *TGLA449*. Additional studies (Schmutz et al., 1995, Harlizius et al., 1997) also found the polled locus to be located in this same region. However, efforts to further refine the location of the polled locus have proved difficult due to its location near the centromere, because few recombinants are found in this region of the chromosome. Song (1998) and Stillwell (1998) generated novel genetic markers from bacterial artificial chromosomes **(BAC**) that were subsequently determined to be near the polled locus. Based upon their research, the location of the polled locus was refined to a 1.7 Mb region between the genes interferon receptor 1 (***IFNAR1)*** and superoxide dismutase 1 (***SOD1)***. Drögemüller et al. (2005a) also used genetic linkage analysis to map the polled locus to a 1 Mb region between the genetic markers *RP42-218J17_MS1* and *BM6438*. The 2 regions described in these studies share ~0.5 Mb of overlap on BTA1q12 with only 2 recombinant animals distinguishing the Drögemüller et al. (2005a) map from the maps constructed by Song (1998) and Stillwell (1998). Gaile (2003) used binary trait (i.e. horned vs. non-horned) mapping to test 12 markers positioned on the proximal half of BTA1 for linkage with the unknown polled locus. He determined that the most likely position of the polled locus was near

BM6438.29 or *RACK17.2C6*, both of which are within the polled interval determined by Song (1998) and Stillwell (1998).

Using genetic linkage analysis, the scurs locus has been mapped near the microsatellite marker *BMS2142* (two-point LOD = 4.21) on BTA19 (Asai et al., 2004). This region lies near the genes *ALOX12* and *MFAP4*. However, in the Texas A&M University Angleton project, the most likely location of the scurs locus was on BTA12 (multi-point LOD = 2.5) near the microsatellite *TEXAN3,* and there was no evidence of a scurs locus on BTA19 (unpublished data).

Polled Intersex Syndrome

In goats, Pailhoux et al. (2001) described a mechanism associated with polled intersex syndrome **(PIS)** where a 11.7 kb deletion affects the long range-transcription of 2 genes, PIS-regulated transcript 1 (***PISRT1***) and Forkhead box L2 (***FOXL2***), resulting in polledness and intersexuality. The *FOXL2* gene is located approximately 200 kb away from the PIS deletion. It is involved in ovarian maintenance and is responsible for the dominantly inherited blepharophimosis ptosis epicanthus inversus syndrome in humans, which results in eyelid malformation and ovarian failure. Located approximately 20 kb from the deletion, *PISRT1* produces a non-coding RNA and does not have a clear open-reading frame.

The sex reversal caused by PIS affects exclusively the XX individuals in a recessive manner, whereas the absence of horns is dominant in both sexes. Pailhoux et al. (2001) found that *PISRT1* is an ‘antitestis’ gene that affects the regulation of the

SOX9 gene, which is a transcription factor that has major roles in both sex determination and in chondrocyte differentiation (Bi et al., 1999). Haploinsufficiency of *SOX9* causes skeletal anomalies. While *PISRT1* and *FOXL2* are not located within the polled critical interval, the PIS mechanism does provide us with clues as to genes that may be involved in the regulation of horn development. Therefore, we predict that the polled gene product may act directly on SOX9 or indirectly through *PISRT1* or *FOXL2*. Our hypothesis is that the polled locus is a tissue specific transcription factor that is expressed in the developing horn buds and acts directly or indirectly upon *SOX9*.

Genes Located in the Polled Interval

The region known to contain the polled locus is located on BTA1q12, which corresponds to HSA21q21-22 and coincides with part of the Down syndrome critical region (Davis et al., 1999). In humans, there are 23 known or predicted genes within this region (Table 1.2). No genes in this interval have direct roles in osteogenesis or chondrogenesis, so there are no obvious candidates for the polled locus. While we have an expectation, based on the observations in polled intersex syndrome, that the polled locus is a transcription factor, genes involved in growth and differentiation, or which interact with genes involved in bone development are candidates as well. The functions of several of the genes from the polled interval are unknown. Based upon what is currently known, the following are candidate genes:

*Oligodendrocyte Lineage Transcription Factors 1 and 2 (**OLIG1 and OLIG2**)*

Each of these genes encodes a basic helix-loop-helix transcription factor expressed in the brain (Jakovcevski and Zecevic, 2005). These transcription factors are involved in the differentiation of the oligodendrocyte lineage, are an essential regulator of neuroectodermal cell fate and may have a role in the learning deficits associated with Down syndrome (NCBI, 2007).

*Chromosome 21 Open Reading Frame 21 (**C21orf66**)*

The *C21orf66* gene encodes a putative transcription factor and alternative splicing results in at least 4 variant isoforms. C21orf66 is a supraspliceosome associated protein having a role in pre-mRNA splicing (Rappsilber et al., 2002; Chen et al., 2007). Expression of this gene appears to be ubiquitous in mouse (MGI, 2007).

*Splicing Factor, Arginine/Serine-Rich 15 (**SFRS15**)*

The SFRS15 protein (also known as KIAA1172, DKFZp434E098, SRA4, and SCAF4) is an arginine/serine-rich pre-mRNA splicing factor, which forms a trimeric protein complex with RNA polymerase II and the tumor protein 63 alpha (TP63α) isoform (Fomenkov et al., 2003; Huang et al., 2005). The N-terminal domain of SFRS15 interacts with the C-terminal domain of RNA polymerase II (Yuryev et al., 1996), while the C-terminal domain interacts with the sterile-α motif at the C-terminus of TP63α (Huang et al., 2005). Failure of the apical ectodermal ridge to differentiate in *TP63* knockout mice causes limbs to be truncated or absent (Mills et al., 1999). Tumor

protein 63 is required for craniofacial and limb development as well as proper skin differentiation because it induces K-SAM and BEK FGFR2 isoforms, which control epithelial and mesenchymal cell fates, respectively (Fomenkov et al., 2003). Fomenkov et al. (2003) proposed that the trimeric protein complex promotes an alternative splicing mechanism leading to the production of the K-SAM FGFR2 isoform essential for epithelial development. They showed that in *TP63* knockout mice, the amount of total FGFR2 mRNA in the skin is not affected but the mesenchymal specific isoform is up-regulated and the alternatively spliced ectoderm-specific isoform is down-regulated. They also demonstrated that mutations in the TP63 sterile-α motif, which abolish binding of TP63 to the SFRS15 and RNA polymerase II complex, prevent alternative splicing of FGFR2 and inhibit epithelial differentiation. Mutations in SFRS15 that affect binding to TP63 or RNA polymerase II may affect bone and skin differentiation via the same mechanism.

*Hormonally Upregulated Neu-Associated Kinase (**HUNK**)*

This protein has an N-terminal catalytic domain typical of serine/threonine kinases (Korobko et al., 2003). Because members of the protein kinase family function as molecular switches in signal transduction pathways that regulate cellular processes such as proliferation and differentiation (Gardner et al., 2000), mutations associated with *HUNK* might prevent horn growth.

*Interleukin 10 Receptor, Beta (**IL10RB**)*

This gene is part of the class II cytokine receptor family (NCBI, 2007). The IL10RB peptide chain (also known as IL10-R2 and CRF2-4) is a required component in the active receptor complexes for interleukin 10 (**IL10**), interleukin 22 (**IL-22**), interleukin 26 (**IL-26**), and interferon-λ (**IFN-λ**) (Donnelly et al., 2004). The binding of these ligands to their respective R1 chains induces a conformational change that enables IL10RB to interact, which in turn activates a signal-transduction cascade. This leads to the rapid activation of several transcription factors, particularly signal transducer and activator of transcription 3 (**STAT3**), STAT1 and genes responsive to these transcription factors.

Interleukin-10 was originally described as a factor produced by T-cells that inhibits cytokine synthesis, but was subsequently shown to be produced by a wide variety of cell types including osteoblasts, macrophages, and keratinocytes (Van Vlasselaer et al., 1994; Roers et al., 2004; García-López et al., 2005). Interleukin-10 suppresses osteoblast differentiation in murine bone marrow cultures by inhibiting the synthesis of transforming growth factor β1, alkaline phosphatase, type I collagen, and osteocalcin (Van Vlasselaer et al., 1994). It also selectively blocks osteoclastogenesis by inhibiting the differentiation of osteoclast progenitors into preosteoclasts (Xu et al., 1995). Knockout mice deficient for IL10 develop osteopenia, decreased bone formation, and mechanical fragility of long bones (Dresner-Pollak et al., 2004). Because interleukins such as IL10 have a known role in bone development and require IL10RB for proper function, *IL10RB* is a candidate for the polled locus.

*Interferon (Alpha, Beta and Omega) Receptors 1 and 2 (**IFNAR1 and IFNAR2**)*

These genes encode type I membrane proteins that form a heterodimeric receptor that is shared by the type I interferon families, Feng, 2005). Both IFNG and IFNB1 are able to modulate osteoclastogenesis (Takahashi et al., 1986; Takayanagi et al., 2002), but IFNG transduces signals via a teterameric IFNGR1/IFNGR2 receptor instead. Interferon-B is part of the autoregulatory pathway that maintains bone homeostasis (Takayanagi et al., 2002), and inhibits c-fos, which is required to form osteoclasts. The effect of IFNB1 on c-fos in osteoclast precursors is mediated by its receptor. Takayanagi et al. (2002) demonstrated that *Ifnar1* $^{-/-}$ mice have increased osteoclastogenesis, but there were no differences in osteoblast differentiation in comparison to the wild type. Thus, mutations in either of the receptor peptide chains could inhibit IFNB1 signaling and disrupt the strict control of osteoclastogenesis thereby affecting horn growth.

Table 1.2. Human genes located in the region on HSA21q21-22 that is homologous to the polled interval on BTA1q12[1]

Gene	Exons	Length (bp)	Gene Type	Status[3]
SOD1	5	9310	Protein coding	Reviewed
SFRS15	20	60718	Protein coding	Provisional
HMG14P	1	575	Pseudogene	Provisional
HUNK	11	130750	Protein coding	Validated
C21orf45	5	10847	Protein coding	Validated
MRAP[2]	5	22972	Protein coding	Validated
SNORA80	1	136	snoRNA	Provisional
C21orf119	1	821	Unknown	Model
C21orf63	8	102946	Protein coding	Validated
C21orf77	1	1452	Unknown	Model
TCP10L	5	8955	Protein coding	Validated
C21orf59	7	10819	Protein coding	Predicted
OR7E23P	1	983	Pseudogene	Provisional
SYNJ1[2]	32	99205	Protein coding	Validated
C21orf66[2]	18	36719	Protein coding	Reviewed
C21orf49	4	26879	Unknown	Model
C21orf62	3	23014	Protein coding	Validated
OLIG2	2	3262	Protein coding	Reviewed
OLIG1	1	2154	Protein coding	Validated
HCG_2045804	4	4625	Unknown	Model
IFNAR2[2]	9	34597	Protein coding	Reviewed
IL10RB	7	30849	Protein coding	Reviewed
IFNAR1	11	34916	Protein coding	Reviewed

[1]NCBI, 2007

[2]Additional known isoforms

[3] Current gene status, based upon NCBI reference sequences (Refseq)

Objectives

A single, dominant mutation is believed to cause the polled phenotype, but the causative gene remains unknown. Prior to this study, genetic mapping had localized the polled locus to a region between the genetic markers *IFNAR* and *SOD1* on BTA1 (Song, 1998; Stillwell, 1998). However, the region between these genes was still largely uncharacterized in cattle. The overall goal of this study was to structurally and functionally characterize this region of BTA1. Specific objectives were to: (1) establish the genomic structure of the polled interval; (2) evaluate *C21orf66* as a positional candidate gene for the polled locus and characterize the genomic structure of this gene in cattle; (3) characterize the expression of genes from the polled interval, as well as other genes with known roles in osteogenesis and chondrogenesis, in neonatal horn buds and polled skin; and (4) determine whether the bony core of the horn develops through intramembranous or endochondral ossification.

CHAPTER II

A 2.5-MB CONTIG CONSTRUCTED FROM ANGUS, LONGHORN, AND HORNED HEREFORD DNA SPANNING THE POLLED INTERVAL ON BOVINE CHROMOSOME 1*

Introduction

The objective of this study was to establish the genomic organization of the polled interval. Potential rearrangements in gene order in comparison to human had been identified using radiation hybrid (**RH**) mapping (Rexroad et al., 1999; Drögemüller et al., 2002), but RH maps often have errors with respect to marker order (Drögemüller et al., 2005). As previously discussed, Song (1998) and Stillwell (1998) mapped the polled locus to the interval between *IFNAR1* and *SOD1* on BTA1 while Drögemüller et al. (2005) mapped the polled locus to a 1 Mb region between the genetic markers *RP42-218J17_MS1* and *BM6438*. We constructed a bacterial artificial chromosome (BAC) contig to encompass both of these regions. This high-resolution physical map of the interval allowed us to compare the arrangement of genes on BTA1 and HSA21, and to accurately position genes and markers in the contig.

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*Reprinted with permission from "A 2.5-Mb contig constructed from Angus, Longhorn and horned Hereford DNA spanning the polled interval on bovine chromosome 1" by Wunderlich K.R., C.A. Abbey, D.R. Clayton, Y. Song, J.E. Schein, M. Georges, W. Coppieters, D.L. Adelson, J.F. Taylor, S.L. Davis, and C.A. Gill, Anim. Genet., 37:592-4, Copyright 2006 by Blackwell Publishing.

Bacterial Artificial Chromosome Contigs

Bacterial artificial chromosomes are a useful tool for construction of high resolution physical maps of mammalian genomes because they have large inserts (100 to 300 kb), high cloning efficiency (10^4 to 10^6 transformants per μg of DNA), and BAC clones are rarely chimeric (Cai et al., 1995; Fairbanks and Anderson, 1999).

A physical map consisting of a contiguous assembly of overlapping BAC clones (i.e. a contig) is more accurate and has better resolution than mapping techniques such as RH mapping (Drögemüller et al., 2005b). Additionally, a BAC contig is a necessary prerequisite for the accurate assembly of whole genome shotgun sequence (Gibbs et al., 2002). Multiple strategies exist for construction of BAC contigs. One approach involves sequencing BAC ends followed by PCR-based screening for additional BAC containing the same sequence. Another involves digesting BAC clones with restriction endonucleases to create BAC fingerprints where overlap between these fingerprints can be identified using a pair-wise search (Luo et al., 2003). A physical map of the bovine genome was recently constructed by BAC fingerprinting (Snelling et al., 2007), while several smaller contigs were constructed using PCR-based methods (Takeda and Sugimoto, 2003; Drögemüller et al., 2005b) or fingerprinting in conjunction with PCR-based methods (Winter et al., 2003).

TAMBT and CHORI-240 BAC Libraries

The TAMBT BAC library (Cai et al., 1995) consisted of 28,000 Angus BAC clones with an average insert size of 146 kb. Later, additional clones were added to the

library and it currently consists of 69,696 Angus BAC clones and 11,328 Longhorn clones (Clare Gill, Texas A&M University, personal communication). To produce this library, DNA was partially digested with *Hind*III and size selected fragments were cloned into the pBeloBAC11 vector as described by Cai et al. (1995). Extracted BAC DNA from individual clones was pooled for PCR-based screening.

The CHORI-240 library (de Jong, 2007) was constructed using DNA from a horned Hereford bull (L1 Domino 99375), which sired the horned Hereford cow (L1 Dominette 01449) used to produce the bovine whole-genome shotgun sequence (http://hgsc.bcm.tmc.edu/projects/bovine/). DNA was partially digested with *Mbo*I and size selected DNA was cloned into the vector pTARBAC1.3. The library consists of approximately 191,737 recombinant clones with an average insert size of 167 kb (http://bacpac.chori.org/bovine240.htm).

Partial BAC Contig of the Polled Interval

Song (1998) and Stillwell (1998) constructed a partial BAC contig of the polled critical interval. To begin, they identified new microsatellite markers in BAC clones containing published markers. These 10 new and published markers (*AGLA17, TAMU199, IFNAR, TAMU202, BM6438, TAMU222, TAMU223, SOD1, SOD1M1*, and *SOD1M2*; Table A.1) were used to isolate 16 Angus BAC clones from the TAMBT library as described by Cai et al. (1995). Overlap between these BAC clones was determined by *Hind*III digestion. Additional BAC were added to the contig by chromosome walking. BAC end sequences (**BES**) were generated and used to design

new primers, which were subsequently used to screen the TAMBT library. Other STS markers were also amplified, and presence or absence of these markers in the BAC clones was used to establish marker order. By this approach, Stillwell (1998) used 42 markers to identify 28 BAC clones between *AGLA17* and *SOD1*. However, the BAC clones were only completely contiguous between the markers *IFNARTACA* and *SYNJ1*, covering approximately 800 kb of the 2.5 Mb region. Large gaps still existed in the contig, and numerous genes within the polled interval needed to be anchored to the contig.

Materials and Methods

BAC End Sequences

The partial BAC contig created by Stillwell (1998) was used as starting point for contig construction. Initially, BAC end sequences (**BES**) that were used to isolate additional, overlapping, Angus BAC clones within the polled critical interval were generated by plasmid end rescue (Cai et al., 1995). Later, BES were generated by direct sequencing of BAC DNA with Big Dye v.3 terminators (Applied Biosystems, Foster City, CA) using SP6 (ATTTAGGTGACACTATAG) or T7 (TAATACGACTCACTATAGGG) primers. These BES were aligned to the human genome sequence using the BLAST algorithm (Altschul et al., 1990; Altschul et al., 1997) to ensure that the sequence aligned to the region of HSA21 homologous to the polled interval. The BES were also aligned to the draft bovine genome sequence but

sequence mis-assembly and unordered scaffolds caused some erroneous sequence placement outside of the polled interval on BTA1.

Primer Design and PCR

The BES as well as sequence data available in the public domain (NCBI, 2007) were used as templates to design primers using Primer v0.5 (Lincoln et al., 1991). Regions of repetitive and low complexity DNA were masked prior to primer design using Repeatmasker software (www.repeatmasker.org). These primers were subsequently aligned against the human or bovine genome sequences using the BLAST algorithm (Altschul et al., 1990; Altschul et al., 1997) to ensure primer binding sites were unique. Various temperatures (~50 to 64°C) and magnesium concentrations (1.5 mM to 3.5 mM) were tested to optimize primers until a single amplicon of the expected size was obtained by PCR (40 ng DNA template, 1X Taq buffer, 1.5-3.5 mM $MgCl_2$, 0.12 mM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer, and 1U Taq polymerase). Thermal cycling was performed on a MJ PTC-200 or GeneAmp PCR System 9700 (MJ Research, Waltham, MA; Applied Biosystems, Foster City, CA) thermal cycler (95°C for 5 min; 35 cycles at 95°C for 30 sec, 50-64°C for 30 sec, 72°C for 30 sec; 72°C for 7 min). Amplicons were separated by size using agarose gel (2% agarose, 1X TAE, 0.2 ug/mL ethidium bromide) electrophoresis and visualized under ultraviolet light (302 nm). Gels were photographed using a Kodak DC290 digital camera with the 1D image analysis software (Eastman Kodak Co., Rochester, NY). These primers and PCR conditions (Table A.1) were then used to screen pooled BAC

DNA from the TAMBT library as described by Cai et al. (1995). Genomic DNA was a positive amplification control, and a template negative control was run for each primer pair.

Addition of BAC Clones From CHORI-240 Library

Angus BAC clones from the TAMBT library and associated mapping data were contributed to the International Bovine BAC Map Consortium (IBBMC; http://www.bcgsc.ca/platform/mapping/bovine; Snelling et al., 2007). TAMBT and CHORI-240 clones were fingerprinted as part of that project. Using the IBBMC fingerprint data, we identified 186 BAC clones from the CHORI-240 library predicted to map to the critical interval using iCE v3.3 (Fjell et al., 2003). A total of 90 of these BAC clones were ultimately part of the contig and, where available, corresponding BES were recovered from GenBank.

Sequence Tagged Site Mapping

Relevant clones from the TAMBT and CHORI-240 libraries were picked into new 96 well plates containing 150 µl LB broth (Invitrogen, Carlsbad, CA; Cat. No. 12795-084) and grown overnight in a rotary shaker at 37° C. These were subsequently mixed with 150 µl 2X freezing media (72 mM K_2HPO_4, 26 mM KH_2PO_4, 3.4 mM sodium citrate, 0.8 mM $MgSO_4$, 13.6 mM $(NH_4)_2SO_4$, 8.8% glycerol) to make glycerol stock plates containing the 186 CHORI-240 and 46 previously and newly identified TAMBT BAC clones needed for screening. These were frozen and stored at -80° C. A

96 prong replicator was later used to inoculate 100 µl of d_2H_2O with the individual BAC clones. Following denaturation for 2 minutes at 95° C, 1 µl was transferred to PCR plates to serve as the template for PCR (Table A.1). This subset of BAC was screened with every primer pair developed for the contig to produce an STS content map. Presence or absence of amplification was noted for each primer pair and clone to order the BAC clones across the region. In total, 30 previously mapped microsatellites or single-strand conformation polymorphisms, 23 gene-specific STS, 22 STS derived from BES and 5 anonymous STS were mapped to the contig. These STS were deposited in Genbank (DQ886274-DQ886354; Table A.1).

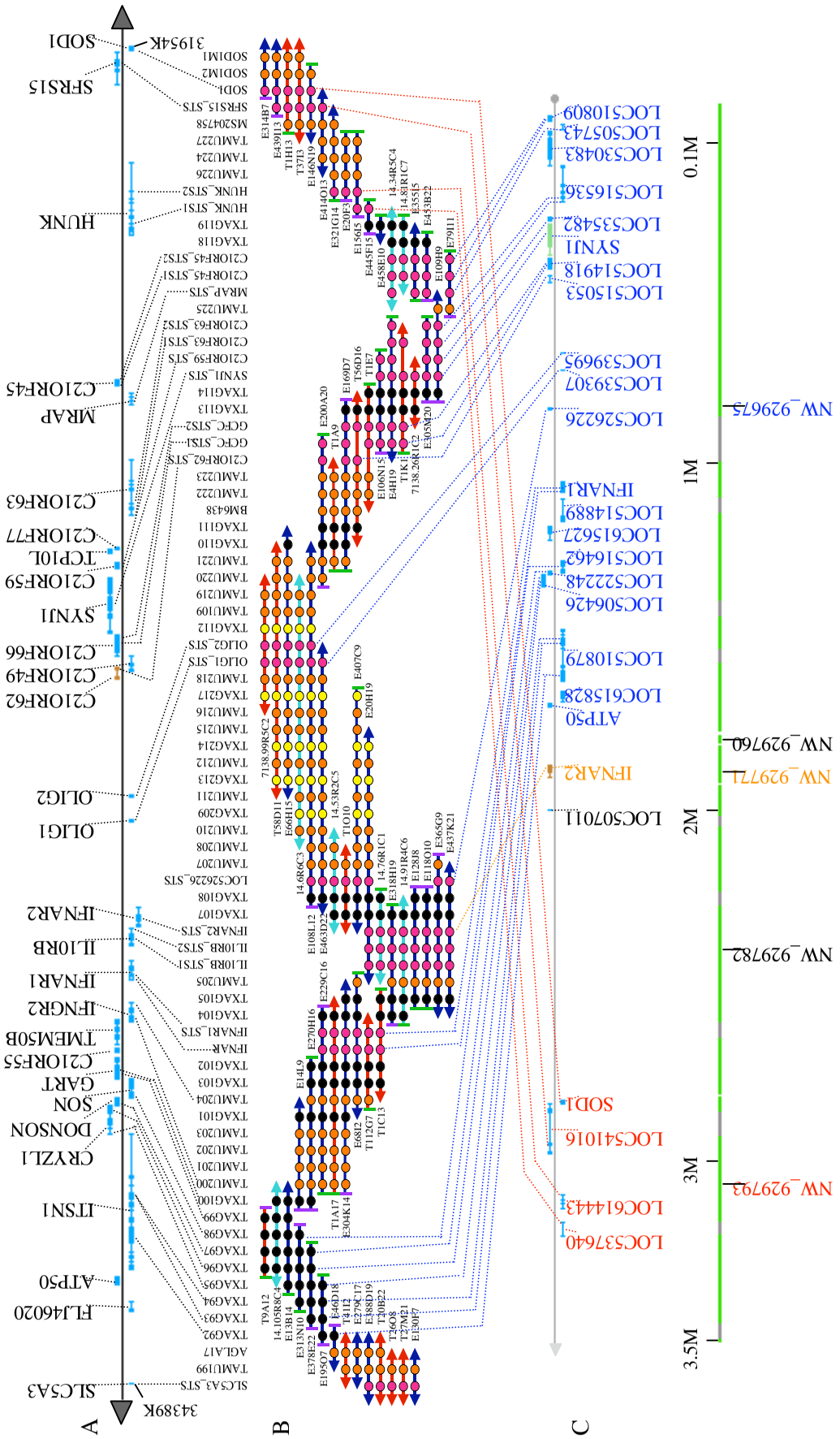
Results and Discussion

A single contig of ca 2.5 Mb, with an average marker spacing of 31.25 kb, was constructed, and 40% of the STS were represented by Hereford and Angus clones; 27.5% by clones from all 3 breeds; 17.5% by Hereford and Longhorn clones; and 15% by only Hereford clones (Wunderlich et al., 2006; Figure 2.1). Alignment of STS and BES to the human genome sequence (Build 36.1) using BLASTN (Altschul et al., 1990) allowed 22 annotated human genes from the corresponding region on HSA21 to be anchored to the physical map, including 7 genes not anchored by Drögemüller et al. (2005a). No rearrangements of genes within this interval were found, which is in agreement with Drögemüller et al. (2005a) but in contrast to earlier results based on radiation hybrid mapping and early assemblies of the human genome sequence (Rexroad et al., 1999; Drögemüller et al., 2002).

Bacterial artificial chromosome end sequences from TAMBT and CHORI-240 clones were aligned to the bovine genome sequence (Build 2.1; http://www.hgsc.bcm.tmc.edu/projects/bovine/) to verify the orientation of BAC clones. Of 157 BES, 105 (66.9%) aligned uniquely to scaffolds on BTA1, while the remainder were repetitive, not yet placed on chromosomes, or shared sequence similarities with scaffolds currently assembled on other chromosomes. Assembly of sequence contigs within scaffolds was mostly consistent with our physical order, but the scaffolds themselves were misassembled (Figure 2.1). This is not surprising because this early assembly of the bovine genome was based solely on whole-genome shotgun sequences. Caution should be used when making evolutionary inferences about gene order until genome assemblies have stabilized.

A 4 Mb contig spanning the polled critical interval (from *KRTAP8P1* to *CLIC6*) was previously published based on Holstein BAC clones from the RPCI-42 library (Drögemüller et al., 2005a). This new contig is unique in that it is tied directly to the bovine genome sequence through the use of the horned Hereford BAC clones from the CHORI-240 library. In addition, the combination of BAC clones from horned and polled breeds makes this new contig a useful resource for SNP discovery and the characterization of positional candidate genes.

Figure 2.1. BAC contig and STS map of the polled critical interval relative to human and bovine sequences. (a) Genes from HSA21 (Build 36.1). (b) BAC contig and STS map with Angus BAC shown as red lines, Longhorn BAC as turquoise lines and horned Hereford BAC as blue lines. Forward (T7) BAC ends are represented by green vertical lines, and reverse (SP6) BAC ends are represented by purple vertical lines. Orange dots indicate a microsatellite or SSCP marker, pink dots are gene-specific STS, black dots are STS derived from BES and yellow dots are anonymous STS. Marker names are indicated above the contig. (c) Predicted genes from BTA 1q12 with scaffolds (green and grey lines) from the bovine genome sequence (Build 2.1). (Wunderlich et al., 2006)


A
B
C
SOD1
SFRS15
HUNK
C21ORF45
MRAP
C21ORF63
C21ORF77
TCP10L
C21ORF59
SYNJ1
C21ORF66
C21ORF49
C21ORF62
OLIG2
OLIG1
IFNAR2
IL10RB
IFNAR1
IFNGR2
TMEM50B
C21ORF55
GART
SON
DONSON
CRYZL1
ITSN1
ATP50
FLJ46020
SLC5A3
LOC510809
LOC505743
LOC530483
LOC516536
LOC535482
SYNJ1
LOC514918
LOC515053
LOC539695
LOC539307
LOC526226
IFNAR1
LOC514889
LOC615627
LOC516462
LOC522248
LOC506426
LOC510879
LOC615828
ATP50
IFNAR2
LOC507011
SOD1
LOC541016
LOC614443
LOC537640
0.1M
1M
2M
3M
3.5M
NW_929675
NW_929760
NW_929771
NW_929782
NW_929793

CHAPTER III

GENOMIC STRUCTURE AND POLYMORPHISMS OF THE BOVINE C21orf66 GENE

Introduction

Polled intersex syndrome (PIS) is known to cause polledness and intersexuality in goats (Pailhoux et al., 2001). The deletion that causes this disease is located on chromosome 1q43 in goats, which is homologous to 3q23 in humans. The polled gene in cattle has been mapped to chromosome 1q12 (Song, 1998; Stillwell, 1998; Drögemüller et al, 2005), which is homologous to chromosome 21q22 in humans, indicating that the polled phenotype is caused by different loci in these 2 species. Transcription of *FOXL2* and *PISRT1* is affected by the PIS deletion, and expression of the transcription factor *SOX9*, which is required for chondrogenesis and osteogenesis, is subsequently affected. Based upon the findings of Pailhoux et al. (2001), the hypothesis for this study was that the polled gene in cattle is a tissue specific transcription factor, expressed in developing horn buds that acts directly or indirectly upon *SOX9*.

Based upon the contig and STS map developed in Chapter II, there are 3 transcription factors (*OLIG1*, *OLIG2*, and *C21orf66*) in the polled interval. Of these 3 genes, only *C21orf66* (also known as *GCFC*) was expressed in the developing horn bud (Chapter IV). The objective of this study was to evaluate *C21orf66* as a positional candidate for the polled locus and characterize the genomic structure of this gene in cattle.

Materials and Methods

Assembly of Breed Panel

A breed panel consisting of DNA from 93 animals from horned and polled breeds of cattle (Angus, Brahman, horned Hereford, Polled Hereford, Ankole, Simmental, and Nellore) was assembled (Tables A.2 and A.3). There were 8 samples per breed, except for Angus (n = 31), Brahman (n = 27), and Nellore (n = 3). The additional samples for Angus and Brahman and all 3 Nellore were from the Texas A&M Angleton project DNA repository (Table A.3).

DNA Extraction From Semen

Briefly, semen straws or vials were thawed, emptied into a 1.5 mL tube and cells pelleted by centrifugation for 5 min at 1000g. Extender was decanted and semen pellets were resuspended in 1.5 mL TNE (1M Tris-HCl pH 8.0, 5N NaCl, 0.5M EDTA pH 8.0). Semen pellets were again pelleted by centrifugation at 1000g, and washed 3 times with TNE. Pellets were then resuspended in 1 ml lysis buffer (80% TNE, 1% SDS, 1 mg/ml proteinase K, 50 mM DTT) and incubated overnight in a 37ºC rotary shaker. The cell lysate was transferred to a 15 mL conical tube and mixed with 1.5 mL lysis dilution buffer (85% TNE, 1% SDS, 50 mM DTT). Samples were extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and twice with an equal volume of chloroform with each extraction followed by centrifugation for 10 minutes at 1200*g*. The aqueous phase was subsequently mixed with 250 µl 3M sodium acetate (pH 5.2) and 5.5 mL 100% ethanol, mixed by inversion and placed at -80ºC for 1 hr or -20ºC

overnight to precipitate the DNA. Centrifugation at 4ºC for 10 min at 1200*g* pelleted the DNA, which was washed with 70% ethanol, and again pelleted by centrifugation. Ethanol was decanted, the pellet was allowed to dry for up to 15 min and the DNA was dissolved in 400 µl TE (0.01M Tris pH 7.5, 1 mM EDTA). Quality and quantity of the DNA was evaluated by electrophoresis as previously described.

Construction of a Reference Sequence

In order to sequence the entire *C21orf66* gene from genomic DNA of horned and polled animals, and because this gene had not previously been sequenced in cattle, we first needed to generate sequence from bovine BAC DNA to identify primer binding sites along the length of the gene by chromosome walking. Because direct sequencing of BAC DNA can be inefficient, we developed *Sau*3AI and *Pst*I subclone libraries of 2 TAMBT BAC (209R7C3 and 98R7C12), known to contain *C21orf66* (Chapter II). Subclones containing *C21orf66* STS were sequenced using PUC forward (5'GTTTTCCCAGTCACGAC) and reverse (5'CAGGAAACAGCTATGACC) primers. This was an iterative process with new sequence used to design new primers to screen the subclone libraries and generate more sequence. If the complete sequence of the subclone was not obtained with the universal primers, then internal sequencing primers were designed.

As this sequencing project was progressing, sequence data began being generated as part of the bovine genome project (http://www.hgsc.bcm.tmc.edu/projects/bovine/). As these sequence data became available, we were able to add BAC sequences from the CHORI-240 library (de Jong, 2007) and whole genome shotgun sequences as well. Utilizing our 2.5 Mb BAC contig and STS map (Chapter II) as a resource, we were able to manually assemble 908 separate sequence fragments using Sequencher v3.0 (Gene Codes, Ann Arbor, MI) into a contig spanning over 150,000 bp. This 'reference' sequence was then used for primer design (Table A.4) using Primer v0.5 (Lincoln et al., 1991) across ~53,000 bp of sequence containing *C21orf66*, as well as its 5' and 3' ends.

Polymerase Chain Reaction

Primers were optimized as described in Chapter II (Figure A.3). Because amplicons were relatively long (~1000 bp), dimethyl sulfoxide (**DMSO**) generally was added to the reactions (~50 ng gDNA template, 1X Taq buffer, 1.5-3.5 mM $MgCl_2$, 0.12 mM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer, 1% v/v DMSO, and 1U Taq polymerase). Typical cycle conditions were 95°C for 5 min; 35 cycles at 95°C for 45 sec, 50-64°C for 1 min, 72°C for 2 min; 72°C for 7 min. For a GC-rich region (GCFC_BP8), PCRX Enhancer solution (Invitrogen, Carlsbad, CA; cat. no. 11495-017) was used (1X PCRx Amplification buffer, 0.2 mM each dNTP, 1.5 mM MgSO4, 0.2 μM forward primer, 0.2μM reverse primer, ~40 ng DNA, 2.5 U Platinum Taq DNA polymerase, 1X PCRx Enhancer solution) with thermal cycles of 95ºC for 5 minutes; 35 cycles of 95ºC for 40 seconds, 57ºC for 30 seconds, 68ºC for 1 minutes; 68ºC for 1 min.

To amplify a highly repetitive 5,715 bp region, Takara ExTaq (Takara Bio Inc., Madison, WI) was used for long PCR, following the manufacturer's instructions. Briefly, reactions included ~200 ng DNA, 1X Ex Taq buffer, 0.4 mM each dNTP, 0.5 μM forward primer, 0.5 μM reverse primer, 2.5 u Takara Ex Taq and cycle conditions were 94ºC for 1 minute; 35 cycles of 94ºC for 30 seconds, 66 ºC for 8 minutes; 66 ºC for 8 minutes. All PCR products were cleaned using the Princeton PSI Ψ Clone PCR 96 kits (Princeton Separations, Adelphia, NJ).

Sequencing

Each sequencing reaction (up to 7.5 μl clean PCR product, 0.5 μM primer) was prepared, denatured at 95ºC for 2 min and allowed to cool on ice for 2 min before 2 μl Big Dye v1.1 terminators (Applied Biosystems, Foster City, CA) were added. Cycle sequencing was performed using a MJ Peltier PTC-200 or GeneAmp PCR System 9700 thermal cycler (MJ Research, Waltham, MA; Applied Biosystems, Foster City, CA) for 25 cycles of 96ºC for 10 sec, 50ºC for 5 sec, 60ºC for 4 min. Sequences were cleaned using Montage Seq_{96} cleanup kits (Millipore, Billerica, MA), dried down at 37ºC overnight, re-suspended in 10 μl deionized formamide and loaded on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Sequence Analysis

Sequences were organized by breed, quality clipped and aligned using Phred and Phrap (Ewing and Green, 1998; Ewing et al., 1998). Single nucleotide polymorphisms (**SNP**) were identified using polyphred and manually verified using Consed 13.0 (Gordon et al., 1998). A SNP was recorded if alternate homozygotes or several heterozygotes were observed and the sequence quality was > Phred 13 (1 error in 20 bases) with flanking sequence > Phred 20 (1 error in 100 bases). A breed consensus sequence was created and compared to the consensus sequences for each of the other breeds in order to identify across breed SNP (i.e. nucleotides fixed for alternate alleles in different breeds).

Annotation of C21orf66 in Bovine

As a part of the bovine genome annotation project, the C21orf66 gene was annotated using the Apollo software (http://www.fruitflyorg/annot/apollo; http://bovinegenome.org/apollo/conf). Protein sequence of the human homolog (UniProt Q9Y5B6) was aligned to the consensus set of computationally predicted bovine genes in the bovine GLEAN database (http://genomes.tamu.edu/bovine/blast/blast.html) by BLAST (Altschul et al., 1990, Altschul et al., 1997). The bovine GLEAN (GLEAN_07925) with the best alignment to the human homolog was then verified to be *C21orf66* by BLAST alignment to proteins from others species. The bovine GLEAN was subsequently uploaded into Apollo using the Chado database and gene options, which allowed visualization and alignment of the predicted exon and intron structure of

C21orf66, as well as bovine EST evidence and protein evidence from various species. The start and stop codon, splice sites, exon structures, and UTR were manually verified.

Results and Discussion

Genomic Structure of C21orf66

Similar to its human homolog, bovine *C21orf66* has 18 exons and spans 30,976 bp (Table 3.1). Variant, alternatively spliced isoforms of this gene have also been reported in human (eg, Slavov et al., 2000; Reymond et al., 2001; Hu et al., 2006) and predicted in mouse (NCBI Entrez Gene, 2007). Although variant isoforms have not been described previously in bovine, there appears to be at least one additional variant as evidenced by 2 expressed sequence tags (DV928310 and EE378204) and a full-length cDNA (BC120358) in Genbank. This alternative transcript appears to consist of 12 exons with a start site in the 3rd exon (9th exon of isoform 1) and is in frame with the primary transcript (Table 3.2).

Table 3.1. Primary transcript of *C21orf66*. Position of exons on scaffold Chr1.3 of the bovine genome sequence (Build 3.1) as determined by annotation with Apollo software, and the respective position of exons within the breed consensus sequence used to assign SNP positions

Exon	Genomic Range[1]	Breed Consensus Range[2]	Genomic Length
1	843538-843895	20113-20470	358
2	845436-845564	22010-22138	129
3	849945-850121	26520-26696	177
4	851666-851887	28241-28462	222
5	852761-852864	29336-29439	104
6	853420-853637	29995-30212	218
7	854151-854340	30726-30915	190
8	857932-858055	34507-34630	124
9	861513-861612	38090-38189	100
10	861699-861814	38276-38391	116
11	863704-863903	40281-40480	200
12	864988-865132	41565-41709	145
13	865792-865913	42369-42490	122
14	866653-866729	43230-43306	77
15	867102-867168	43679-43745	67
16	871196-871342	47773-47919	147
17	872973-873127	49550-49704	155
18	874388-874505	50965-51082	118

[1]Relative to position on scaffold Chr1.3

[2]Appendix B

Table 3.2. Exons of a putative alternative transcript of *C21orf66.* Position of exons on scaffold Chr1.3 of the bovine genome sequence (Build 3.1) as determined by annotation with Apollo software, and the respective position of exons within the breed consensus sequence used to assign SNP positions

Exon	Genomic Range[1]	Breed Consensus Range[2]	Genomic Length
1	854021-854340	30596-30915	320
2	857932-858072	34507-34647	141
3	861513-861612	38090-38189	100
4	861699-861814	38276-38391	116
5	863704-863903	40281-40480	200
6	864988-865132	41565-41709	145
7	865792-865913	42369-42490	122
8	866653-866729	43230-43306	77
9	867102-867168	43679-43745	67
10	871196-871342	47773-47919	147
11	872973-873127	49550-49704	155
12	874388-874505	50965-51082	118

[1]Relative to position on scaffold Chr1.3

[2]Appendix B

C21orf66 SNP Discovery

A nearly completely contiguous sequence (~99% complete) of *C21orf66*, its promoter region, and its 3' end, spanning 52,978 bp was assembled for each breed. In some cases, repetitive regions were only represented by sequence from Angus (209R7C3 and 98R7C12) and horned Hereford BAC DNA (E169D7 and E507O6).

There were 144 SNP with a minor allele frequency > 0.05 in at least one breed identified in and around *C21orf66*. The average spacing between SNP was 363 bp. The ratio of transitions to transversions was 2.6:1, which is concordant with the 2:1 ratio found in the bovine HapMap project (Clare Gill, Texas A&M University, personal communication), and that found in human and most other species (Wang et al., 1998). Additionally, 7 insertions or deletions (indels) were identified. Among these 144 SNP, 47 were located 5' to the start site of *C21orf66*, 6 were located in coding sequences, 81 were located within introns, and 10 were located in the 3' untranslated region (Table 3.3). Among the 6 SNP located in the coding region of *C21orf66*, all 6 appear to be silent mutations in either the primary or alternatively spliced transcripts.

Within individual breeds, 98 SNP were discovered in Ankole, 49 in Angus, 41 in Brahman, 39 in Polled Hereford, 34 in Simmental, 29 in horned Hereford, and 27 in Nellore. While a large number of SNP were found in the Ankole, these were almost entirely attributed to a single animal (AK3) that was an alternate homozygote at several SNP positions. The haplotypes for this animal were more similar to *Bos indicus* than to *Bos taurus* animals. While Ankole is considered a *Bos taurus* breed, there has been introgression with *Bos indicus* (Olivier Hanotte, ILRI, Kenya, personal communication).

If Ankole were excluded, there were 68 SNP that were specific to *Bos indicus (Brahman and Nellore)* and 53 SNP that were specific to *Bos taurus* (Angus, horned Hereford, Polled Hereford, and Simmental) breeds.

Causative Mutation

By definition, positions that vary in homozygous polled animals or SNP that are shared among horned and polled animals can be eliminated from consideration as the causative mutation. There was no single position in the *C21orf66* sequence that was completely concordant with the horned and polled phenotypes (in particular, between Angus and horned Hereford). However, this does not rule out *C21orf66* as a candidate gene because it is possible that different mutations cause the polled phenotype in different breeds. Additionally, a long range mutation, beyond the sequenced region, that acts upon *C21orf66* could also cause the polled phenotype. In PIS, the deletion was located 20 kb and 200 kb away from the genes that it affected (Pailhoux et al., 2001). Although the causative mutation was not identified, a large number of novel SNP were identified that will be utilized in future linkage disequilibrium and association analyses.

Table 3.3. Single nucleotide polymorphisms discovered within and around *C21orf66*. The frequency of the minor allele in the sequenced animals is indicated for Angus (AN; n = 23), horned Hereford (HH; n = 8), Polled Hereford (PH; n = 8), Ankole (AK; n = 8), Brahman (BR; n = 19), Nellore (NE; n = 3), and across all breeds

			Bos taurus[1]					***Bos indicus***[1]		
Position[2]	**SNP**[3]	**Location**	**AN**	**HH**	**PH**	**SM**	**AK**	**BR**	**NE**	**All**
1720	GT	5' Region	0.16	0.00	0.00	0.17	0.17	0.10	NA	0.11
2064	GA	5' Region	0.11	0.08	0.00	0.25	0.75	0.10	0.17	0.16
2090	GA	5' Region	0.00	0.07	0.00	0.14	0.14	0.02	0.00	0.04
2163	GT	5' Region	0.12	0.14	0.00	0.43	1.00	0.76	0.67	0.43
2315	CT	5' Region	0.13	0.00	0.60	0.19	0.00	0.00	0.00	0.10
2457	GA	5' Region	0.76	0.86	0.50	0.36	0.00	0.23	0.33	0.47
2663	CT	5' Region	0.15	0.07	0.00	0.36	0.56	0.13	0.33	0.20
2872	AG	5' Region	0.00	0.07	0.00	0.13	0.14	0.05	0.00	0.04
2912	CT	5' Region	0.73	0.86	0.50	0.38	0.00	0.28	0.33	0.48
2994	TC	5' Region	0.00	0.07	0.00	0.00	0.29	0.08	0.17	0.06
3193	AG	5' Region	0.00	0.00	0.00	0.00	0.13	0.02	0.00	0.02
3795	TC	5' Region	0.00	0.07	0.00	0.00	0.17	0.02	NA	0.03
3818	GA	5' Region	0.13	0.00	0.43	0.00	0.00	0.00	NA	0.08
3975	GA	5' Region	0.13	0.00	0.43	0.00	0.00	0.00	NA	0.08
4137	TC	5' Region	0.00	0.00	0.00	0.00	0.14	0.03	0.50	0.03
4955	AG	5' Region	0.00	0.00	0.00	0.00	0.13	0.02	0.25	0.03
5003	TC	5' Region	0.00	0.00	0.00	0.00	0.14	0.02	0.25	0.03
5068	TC	5' Region	0.00	0.00	0.00	0.00	0.14	0.04	0.25	0.03
5569	TC	5' Region	0.00	0.08	0.00	0.21	0.50	0.00	0.00	0.06
7489	AG	5' Region	0.00	0.06	0.00	0.20	0.08	0.02	0.00	0.03
7496	CT	5' Region	0.11	0.00	0.00	0.10	0.00	0.10	0.00	0.07
7730	TC	5' Region	0.00	0.00	0.00	0.00	0.19	0.02	0.17	0.03
7834	TC	5' Region	0.14	0.00	0.50	0.19	0.00	0.00	0.00	0.10
7860	TC	5' Region	0.00	0.00	0.00	0.00	0.13	0.02	0.17	0.02
7894	CT	5' Region	0.76	0.88	0.50	0.38	0.00	0.23	0.33	0.48
7896	CT	5' Region	0.76	0.88	0.50	0.38	0.00	0.23	0.33	0.48
8412	GA	5' Region	0.00	0.00	0.00	0.00	0.13	0.02	0.17	0.02
8803	GA	5' Region	0.14	0.00	0.00	0.21	0.06	0.08	NA	0.09
8913	GA	5' Region	0.00	0.00	0.00	0.00	0.19	0.67	NA	0.23
9049	GA	5' Region	0.12	0.00	0.00	0.25	0.10	0.14	NA	0.11
9284	GA	5' Region	0.13	0.17	0.00	0.50	1.00	0.80	NA	0.45
9476	GC	5' Region	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.01
9480	GA	5' Region	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.01
9729	GA	5' Region	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.01
10504	GA	5' Region	0.75	0.83	0.58	NA	0.00	0.19	NA	0.49

Table 3.3. Continued

			Bos taurus					*Bos indicus*		
Position	**SNP**	**Location**	**AN**	**HH**	**PH**	**SM**	**AK**	**BR**	**NE**	**All**
10623	CT	5' Region	0.74	0.83	0.58	NA	0.00	0.18	NA	0.48
10653	CT	5' Region	0.00	0.08	0.00	NA	0.17	0.02	NA	0.03
15334	AG	5' Region	0.00	0.00	0.00	0.00	0.19	0.57	NA	0.18
16813	GT	5' Region	0.73	0.88	0.57	0.25	0.00	0.20	NA	0.47
18162	AC	5' Region	0.00	NA	NA	0.00	0.00	0.08	0.50	0.04
18608	GT	5' Region	0.00	0.00	0.00	0.00	0.13	0.00	NA	0.01
18734	TC	5' Region	0.16	0.00	0.50	0.21	0.19	0.64	0.50	0.32
18770	GA	5' Region	0.00	0.00	0.00	0.00	0.13	0.02	0.00	0.02
18843	GA	5' Region	0.00	0.00	0.00	0.00	0.13	0.02	0.00	0.02
18938	TC	5' Region	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.01
18956	AC	5' Region	0.12	0.00	0.42	0.36	0.31	0.69	0.50	0.30
18972	CT	5' Region	0.08	0.00	0.50	0.21	0.19	0.67	NA	0.25
20915	TG	Intron 1	0.20	0.00	0.50	0.00	0.00	0.00	NA	0.12
23085	GC	Intron 2	1.00	0.80	0.50	0.33	0.00	0.18	0.00	0.38
23086	CG	Intron 2	0.00	0.00	0.00	0.00	0.00	0.35	0.00	0.13
23902	CT	Intron 2	0.09	NA	0.42	NA	NA	0.00	NA	0.11
24145	GA	Intron 2	0.12	0.25	0.00	0.44	0.50	0.75	0.25	0.39
24383	TC	Intron 2	0.18	0.00	0.50	0.13	0.80	0.03	0.25	0.19
24388	AT	Intron 2	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.01
24917	CG	Intron 2	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.01
25475	CT	Intron 2	0.00	0.00	0.00	0.00	0.17	0.02	0.00	0.02
26276	TC	Intron 2	0.23	0.00	0.75	NA	NA	0.00	0.00	0.13
26954	AT	Intron 3	0.18	0.17	0.40	NA	0.88	0.15	0.00	0.26
27036	TG	Intron 3	0.29	0.00	0.38	NA	0.25	0.04	0.00	0.14
27531	TC	Intron 3	0.17	0.00	0.42	0.13	0.20	0.02	0.00	0.12
27613	GA	Intron 3	0.14	0.00	0.42	0.13	0.00	0.00	0.00	0.09
28929	GT	Intron 4	0.18	0.00	0.60	0.30	0.25	0.03	0.25	0.18
29916	TG	Intron 4	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.01
30015	AG	Exon 6	0.05	0.00	0.33	0.00	0.00	0.02	0.17	0.05
30108	CT	Exon 6	0.09	0.00	0.33	0.00	0.00	0.00	0.00	0.05
30398	CT	Intron 6	0.12	0.13	0.00	0.88	1.00	0.82	0.50	0.43
31116	CT	Intron 7	0.00	0.00	0.00	0.00	0.14	0.02	0.00	0.02
31266	GA	Intron 7	0.15	0.00	0.43	0.19	0.00	0.00	0.00	0.10
31449	TC	Intron 7	0.16	0.00	0.50	0.14	0.00	0.00	0.00	0.10
31564	AC	Intron 7	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.04
31794	TC	Intron 7	0.15	0.00	0.50	0.17	0.00	0.00	0.00	0.10
32111	GT	Intron 7	0.00	0.00	0.00	0.07	0.38	0.00	0.00	0.04
33212	TC	Intron 7	0.15	0.00	0.42	0.14	0.17	0.02	0.00	0.11
34112	AG	Intron 7	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.02
34279	CT	Intron 7	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.02

Table 3.3. Continued

			Bos taurus					*Bos indicus*		
Position	**SNP**	**Location**	**AN**	**HH**	**PH**	**SM**	**AK**	**BR**	**NE**	**All**
36069	AG	Intron 8	0.00	0.00	0.00	0.00	0.13	0.02	0.17	0.02
36415	CA	Intron 8	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.01
36460	TC	Intron 8	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.01
36683	AG	Intron 8	0.00	0.00	0.00	0.00	0.13	0.00	0.17	0.02
37393	TG	Intron 8	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.01
37412	CT	Intron 8	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.01
37413	TG	Intron 8	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.01
37484	AG	Intron 8	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.01
37505	TC	Intron 8	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.01
37511	AG	Intron 8	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.01
37540	TC	Intron 8	0.00	0.00	0.00	0.00	0.25	0.00	0.25	0.02
37615	TG	Intron 8	0.00	0.00	0.00	0.00	0.25	0.00	0.25	0.02
37812	CG	Intron 8	0.15	0.00	0.43	0.19	0.00	0.00	0.00	0.10
38363	GA	Exon 10	0.00	0.00	0.00	0.00	0.13	0.02	0.00	0.02
38372	AG	Exon 10	0.12	0.06	0.00	0.25	0.19	0.08	0.17	0.11
38485	TC	Intron 10	0.13	0.00	0.43	0.19	0.13	0.02	0.17	0.11
38551	CT	Intron 10	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.01
38647	TC	Intron 10	0.00	0.00	0.00	0.00	0.19	0.60	0.33	0.20
38775	CA	Intron 10	0.12	0.00	0.38	0.19	0.00	0.00	0.00	0.08
38793	GA	Intron 10	0.00	0.00	0.00	0.00	0.13	0.02	0.00	0.02
38804	CA	Intron 10	0.00	0.00	0.00	0.00	0.13	0.02	0.17	0.02
39082	AG	Intron 10	0.00	0.06	0.00	0.14	0.60	0.02	0.00	0.06
39461	CT	Intron 10	0.15	0.00	0.43	0.21	0.25	0.02	0.17	0.13
40012	CA	Intron 10	0.00	0.00	0.00	0.00	0.14	0.00	0.17	0.02
40037	TC	Intron 10	0.12	0.07	0.00	0.21	0.21	0.09	0.17	0.11
40471	TG	Exon 11	0.00	0.00	0.00	0.00	0.14	0.02	0.00	0.02
40783	GA	Intron 11	0.00	0.00	0.00	0.00	0.13	0.02	0.00	0.02
40825	AG	Intron 11	0.00	0.00	0.00	0.00	0.13	0.02	0.17	0.02
41362	GC	Intron 11	0.26	0.00	0.43	0.33	0.19	0.11	0.33	0.21
41368	TC	Intron 11	0.00	0.00	0.00	0.00	0.81	0.65	0.33	0.28
41387	CG	Intron 11	0.00	0.00	0.00	0.00	0.13	0.02	0.17	0.02
41451	AG	Intron 11	0.00	0.00	0.00	0.00	0.13	0.02	0.17	0.02
41693	TC	Exon 12	0.10	0.00	1.00	NA	0.00	0.00	NA	0.07
41826	GA	Intron 12	0.00	0.00	0.00	NA	0.13	0.00	0.00	0.02
42311	AG	Intron 12	0.00	0.00	0.00	NA	0.14	0.00	0.17	0.03
42571	CT	Intron 13	0.17	NA	NA	0.06	NA	0.00	NA	0.07
42709	GA	Intron 13	0.00	NA	NA	0.13	NA	0.07	NA	0.07
42902	AC	Intron 13	0.00	NA	NA	0.17	NA	0.00	0.00	0.02
42906	TC	Intron 13	0.00	NA	NA	0.25	NA	0.00	0.00	0.03

Table 3.3. Continued

			Bos taurus					*Bos indicus*		
Position	**SNP**	**Location**	**AN**	**HH**	**PH**	**SM**	**AK**	**BR**	**NE**	**All**
43378	TG	Intron 14	0.00	0.00	0.00	0.00	0.13	0.02	0.17	0.02
43412	TC	Intron 14	0.00	0.00	0.00	0.00	0.86	0.00	0.17	0.10
43491	CT	Intron 14	0.00	0.00	0.00	0.00	0.14	0.02	0.17	0.02
44492	TC	Intron 15	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.01
44717	TC	Intron 15	0.00	NA	NA	0.06	NA	0.00	0.00	0.01
45769	TC	Intron 15	0.69	0.86	0.57	0.50	0.00	0.22	0.33	0.46
46527	GA	Intron 15	0.16	0.00	0.43	0.19	0.00	0.00	0.00	0.11
47229	AC	Intron 15	0.12	0.00	0.00	0.29	0.07	0.04	0.25	0.09
47977	TC	Intron 16	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.01
48625	GC	Intron 16	0.00	0.00	0.00	0.00	0.10	0.63	0.33	0.21
49172	GA	Intron 16	0.48	0.00	0.00	0.50	0.00	0.00	0.00	0.20
49815	AT	Intron 17	0.73	0.90	0.58	0.40	0.00	0.23	0.33	0.47
49840	GA	Intron 17	0.00	0.00	0.00	0.00	0.17	0.02	0.17	0.03
49898	AG	Intron 17	0.00	0.00	0.00	0.00	0.08	0.60	0.33	0.22
49962	AC	Intron 17	0.00	0.00	0.00	0.00	0.17	0.02	0.17	0.03
50015	GA	Intron 17	0.00	0.00	0.00	0.00	0.08	0.60	0.33	0.22
50564	GA	Intron 17	0.00	0.00	0.00	0.00	0.13	0.68	0.33	0.24
50588	AG	Intron 17	0.23	0.00	0.43	0.13	0.25	0.12	0.33	0.19
50751	CG	Intron 17	0.00	0.00	0.00	0.00	0.25	0.02	0.17	0.03
50931	CT	Intron 17	0.00	0.00	0.00	0.00	0.33	0.02	0.17	0.03
52061	CA	3' UTR	0.00	0.00	0.00	0.00	0.25	0.03	0.25	0.03
52150	TG	3' UTR	0.27	0.00	0.43	0.13	0.00	0.08	0.00	0.16
53132	CT	3' UTR	0.00	0.00	0.00	0.00	0.17	0.02	0.17	0.02
53580	GC	3' UTR	0.00	0.00	0.00	0.00	0.17	0.02	0.17	0.02
53624	GA	3' UTR	0.16	0.00	0.50	0.00	0.00	0.00	0.00	0.08
53701	TC	3' UTR	0.00	0.00	0.00	0.13	0.25	0.00	0.00	0.02
53816	CT	3' UTR	0.00	0.17	0.00	0.00	0.25	0.02	0.17	0.05
53851	AG	3' UTR	0.00	0.00	0.00	0.00	0.25	0.02	0.17	0.03
53862	GA	3' UTR	0.00	0.00	0.00	0.00	0.25	0.02	0.00	0.02
54081	GC	3' UTR	0.00	0.00	0.00	0.00	0.25	0.02	0.17	0.03

[1]NA = No data available

[2]Position in breed consensus sequence (Appendix B)

[3]Most frequently observed allele in the breed panel (n = 93) is listed first

CHAPTER IV

QUALITATIVE AND QUANTITATIVE ASSESSMENT OF GENE EXPRESSION IN HORN BUDS AND POLLED SKIN

Introduction

Understanding patterns of gene expression is expected to provide insight into complex regulatory networks and will likely lead to the identification of genes relevant to new biological processes or implicated in disease (Vandesompele et al., 2002). Among the 23 genes located within the interval from *IFNAR1* to *SOD1* on BTA1 (NCBI, 2007), there is not an obvious candidate gene for the polled phenotype. While some of these genes do have roles associated with growth (*HUNK*) or bone development (*SFRS15, IL10RB, IFNAR1,* and *IFNAR2*) that could suggest a role in horn development, there are also several genes within the region whose function is unknown (*C21orf63, C21orf45, C21orf119, C21orf77, C21orf59, C21orf66, C21orf49,* and *C21orf62*). In order to better establish which of these genes could be the locus causing the polled phenotype, it needs to be determined if the gene is actually expressed within the bovine horn or in skin from the head of a polled animal sampled from the location where a horn would be expected to grow.

Objective

The objective of this study was to characterize the expression of genes from the polled interval (*IFNAR1* to *SOD1* on BTA1), as well as other genes with known roles in

osteogenesis and chondrogenesis, in neonatal horn buds and polled skin. While the exact time of initiation of horn development is unknown, Lange et al. (1990) stated that horn tissue is present in a 3 mo old calf fetus. In newborn calves, a very small horn may be present. Because the horn is in the early stages of development at this point and horn growth occurs primarily after birth, we hypothesized that the polled gene would be expressed in the horn buds or polled skin of newborn calves. Additionally, we would expect other early regulators of bone development to be expressed at this time point. In order to refine the list of candidate genes for polled, the presence or absence of expression of genes located within the polled interval was investigated by reverse transcriptase-PCR (**RT-PCR**). Subsequently quantitative real-time RT-PCR (**qRT-PCR**) was used to determine which of these genes were differentially expressed among horned, scurred, and polled calves. It is expected that the polled gene will be differentially expressed between a horned and polled animal. Gene expression at 2 different time points (~1 d and ~5 mo) was evaluated to characterize changes in expression in the developing horn, scur or skin.

Potential Regulators

There are a vast and growing number of genes with known roles in chondrogenesis and osteogenesis. Several gene families and developmental pathways are known to be involved, including *RUNX, SOX, COL, WNT, TWIST, FGF, HH, PTH, STAT,* and *BMP* family members. For example, the *RUNX* transcription factors are involved in chondrogenesis where RUNX2 and RUNX3 control chondrocyte maturation,

and RUNX2 is a major regulator in both endochondral and intramembranous ossification through osteoblast differentiation (Flores et al., 2006). The SRY-box (*SOX*) transcription factors (*L-SOX5*, *SOX6*, and *SOX9*) function in cartilage development and are required for expression of other factors such as *COL9A1*, *AGC1*, *COL2A1* and *COL11A2* (Bi et al., 1999;Goldring et al., 2006). These precollagens (*COL*) are required for the differentiation of chondroprogenitors (COL2A1, COL9A2, and COL11A2) and are involved in the subsequent matrix calcification (COLX) and ossification (COLII) during endochondral ossification (Goldring et al., 2006). The expression patterns of many *WNT* genes during skeletal development suggests that they may signal to the mesenchymal condensations and regulate osteoblast and chondrocyte differentiation and are likely upstream regulators of the transcription factors *RUNX2*, *SOX9*, and Osterix (*OSX*) (Day et al., 2005). The TWIST proteins (TWIST1 and TWIST2) are basic helix-loop-helix (bHLH) containing transcription factors that are expressed in the sutures and inhibit RUNX2 in skeletogenesis (Bialek et al., 2004). The fibroblast growth factors (*FGF*) include genes that act as patterning molecules early in development and later are essential positive and negative regulators of bone development (Karsenty, 2001a). They are known to interact with SOX9, STAT1, and IHH, which are also involved in bone development (Kronenberg, 2003). Among the hedgehog homologs (*HH*), IHH is produced by pre-hypertrophic and hypertrophic chondrocytes and participates in a negative feedback loop with PTHLH that synchronizes and determines the pace of differentiation of chondrocytes in the growth plate (Kronenberg, 2003). Sonic hedgehog (SHH) mediates its effects on bone development by inducing SOX9 expression (Nie et

al., 2005). The *PTH* genes are involved in bone mineralization. More specifically, PTHLH maintains chondrocytes in a proliferative, less differentiated state where deficient mice have abnormal endochondral bone formation (Kronenburg and Chung, 2001). The *STAT* genes are part of the Jak-Stat pathway involved in osteoblast and osteoclast differentiation and are important in FGF modulation (Xiao et al, 2004; Itoh et al, 2006). Bone morphogenetic protein (BMP) signaling is necessary for the formation of precartilaginous condensations, their differentiation into chondrocytes, and for maintenance of chondrogenesis. Data suggest that *BMP* are master genes able to trigger the entire series of events needed for normal chondrogenesis (Pizette and Niswander, 2001). The roles of some of these gene family members during endochondral and intramembranous ossification are schematically represented in Chapter I (Figures 1.2 and 1.3).

Materials and Methods

Primer Design

Sequences for genes in the polled interval from *IFNAR1* to *SOD1* on BTA1, as well as selected other genes with known roles in chondrogenesis and osteogenesis were obtained from NCBI Gene (NCBI, 2007). In silico predicted bovine mRNA sequences or known bovine mRNA sequences were compared to the annotated human genome sequence using the BLAST algorithm (Altschul et al., 1990, Altschul et al., 1997) to ensure that orthologous genes were being investigated. For bovine mRNA sequences that did not align to the correct human gene, or those genes where no mRNA sequences

were listed for the bovine gene, human mRNA sequences were used for BLAST analysis of the bovine expressed sequence tag (**EST**) database. Bovine EST were aligned to the bovine genome sequence, and apparent exons and introns were identified.

Primers for selected sequences were designed for qualitative RT-PCR with Primer v0.5 (Lincoln et al., 1991). Primers were preferentially designed to span introns <1000 bp in length when possible. Genomic DNA and RNA were used as controls. Amplicons ranged from 61 to 1825 bp (Table A.5) in genomic DNA and 61 to 664 bp in cDNA.

Gene specific primers for qRT-PCR (Table A.6) were designed using Primer Express software (Applied Biosystems, Foster City, CA). Products ranged from 52 to 185 bp with a melting temperature from 58 to 60ºC. When possible, primers were designed to span an intron. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Tissue Collection

All procedures involving animals were approved by the Texas A&M Institutional Animal Care and Use Committee. For the qualitative RT-PCR experiments, equal numbers of bull and heifer calves were identified from herds at the McGregor Research Station with breeding that would produce horned, polled and potentially scurred animals. Neonatal horn buds (n = 10), skin from putatively scurred calves (n=12), and skin from polled calves (n = 8) were collected at one day of age using a tube dehorner. Excess hair was removed with clippers, and the skin prepared in an aseptic fashion using

chlorhexidine scrub and isopropyl alcohol. The tube was placed over the base of the horn (or in an equivalent location on polled or putatively scurred animals) so that approximately 6.5 mm of skin around the base of the horn was included within the tube. The tube was pushed and twisted each way until the skin was cut to a depth of 3 to 9 mm, then twisted inward and downward (towards the jaw) to cut under the horn button and spoon it out. Blood coagulation powder and fly spray were used to treat the resultant wounds. Both sides of the head were sampled, with one sample stored in RNALater (Ambion, Austin, TX) and the other fixed in 4% paraformaldehyde.

Additional tissues were collected for qRT-PCR after initial qualitative experiments. Horn buds or polled skin from the left side of the head were collected when calves were 1 to 8 d old. The other horn, scur, or polled skin was collected when these calves were 5 to 6 mo of age or at 12 mo of age (data not shown). Because scurs are not evident on calves less than one mo old, calves were not identified as scurred until the second biopsy date. At each collection, polled skin samples were collected from 5 male and 5 female Angus calves, horned samples from 5 male and 5 female Nellore calves or 8 male and 8 female calves capable of being horned based upon breed type (Figure A.7), and putative scurs were collected from up to 8 male and 8 female calves that were heterozygous at the polled locus (½ Angus, ½ Brahman or ½ Angus, ½ Nellore). Tissue biopsies were obtained in the field. For polled calves and animals with small horns or scurs, biopsies were collected with a tube dehorner as previously described, then placed into RNALater (Applied Biosystems, Foster City, CA, USA). At 5 to 6 mo of age, horn and scur samples that were too large for a tube dehorner were

collected with a scoop dehorner, snap frozen in liquid nitrogen, and transferred to -80ºC. Putative horns and scurs were distinguished by SNP genotyping and haplotype analysis that verified the breed of origin of DNA in the polled interval.

Additional tissues (liver, lung, heart, spleen, muscle, brain, and kidney) were collected from steers during slaughter at the Rosenthal Meat Science and Technology Center in College Station. These steers were all a part of the McGregor Genomics Project, and were approximately 18 mo old at the time of slaughter. Samples were collected as soon as possible, but due to the nature of the process, there was generally 30 to 60 min between time of death and sample collection that could not be avoided. Tissue samples were chopped into small pieces using a razor blade, placed into screw-cap tubes, and snap frozen in liquid nitrogen. Samples were then transferred to -80ºC for storage.

RNA Extraction

RNA was extracted in Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, approximately 0.5 g tissue was cut into small pieces using a sterile razor blade and quickly transferred to a 50 mL conical tube containing 5 mL Trizol reagent (Invitrogen, Carlsbad, CA). These samples were mechanically homogenized (Tissue Tearor model 398, Biospec Products Inc.) at a speed of 20 rpm for 30 sec. The homogenizer was cleared of tissue debris and rinsed twice in DEPC-treated water. Homogenization was then repeated at speeds of 25 rpm and 30 rpm. Samples were transferred to clean tubes for centrifugation (Sorvall RC5C, SM-24 rotor, Newtown, CT) at 12000g for 10 minutes at 4ºC. The supernatant was transferred

to a new tube, and allowed to incubate for 5 min at room temperature followed by extraction with 1 mL chloroform. After centrifugation at 4ºC for 10 min at 12,000g, the upper aqueous phase was transferred to a clean tube. RNA was precipitated with one volume of isopropanol and washed with 70% ethanol. Ethanol was decanted and the pellet was allowed to air dry for 5 to 10 min. Samples were re-suspended in 150 to 200 µl DEPC-treated water (Ambion, Austin, TX), vortexed briefly, heated to 55 ºC for 5 min, and again vortexed.

Total RNA was quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Replicate readings were averaged. RNA quality for the qualitative PCR experiments was assessed by formaldehyde gel electrophoresis (1% agarose, 1% Northern running buffer, 5% v/v of 37% formaldehyde) in Northern running buffer (20 mM MOPS, 1 mM EDTA, 5 mM Na Acetate, pH 7.0). A 2 µl aliquot of RNA was mixed with 1.6 µl of 10X formaldehyde gel loading buffer (50% glycerol, 10 mM EDTA pH 8, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol) and combined with sample loading buffer (50% v/v deionized formamide, 18% v/v of 37% formaldehyde, 0.1 ug/ul ethidium bromide, 1% Northern running buffer). This mixture was denatured at 70ºC for 10 min, chilled on ice, and loaded onto the formaldehyde agarose gel. After electrophoresis at 110 V for 35 min, the 28S and 18S bands were visualized under UV light (302 nm). RNA samples were stored at -80ºC. Total RNA sample quality for qRT-PCR was assessed by capillary electrophoresis on an RNA 6000 NanoChip with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto,

California). Degraded RNA samples were excluded from further analysis (Fleige and Pfaffl, 2006).

RNA samples were treated with RQ1 DNase (Promega, Madison, WI) according to the manufacturer's instructions to remove any contaminating DNA. However, to stop the reaction, 13 mM EDTA was substituted for the stop solution to minimize the inhibitory effect on subsequent reactions caused by the stop solution. Following DNase treatment, RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Reverse Transcriptase Polymerase Chain Reaction

Reverse transcription was performed using the SuperScript First Strand Synthesis System for RT-PCR with Oligo(dT) primers (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. DNase treated RNA samples (0.5 μg) were used as the template for the reaction. Following reverse transcription, 1 μl of the newly synthesized cDNA was used as a template for PCR (Chapter II). Genomic DNA, an RT negative, and a template negative control were included for each set of PCR reactions. Additionally, an RNA control was run for each sample if the amplicon was from a single exon to ensure that amplification was not the result of DNA contamination. Amplicons were visualized under UV light (302nm) following agarose gel (2% agarose, 1X TAE, 0.2 μg/mL ethidium bromide) electrophoresis. Presence or absence of gene expression was qualitatively determined as the ability to visualize a band on these agarose gels. The

PCR experiment was duplicated, although new cDNA was often generated to perform the duplicate PCR.

Real-Time Polymerase Chain Reaction

RNA samples were reverse transcribed into cDNA as previously described, and reactions were primed with random hexamers or gene-specific primers instead of oligo(dT) and the reactions were incubated at 25ºC for 10 minutes, 42ºC for 50 minutes, and 70ºC for 15 minutes. These cDNA samples were then used as a template for qRT-PCR utilizing SYBR Green PCR mix (Applied Biosystems, Foster City, CA). Each 10 µl reaction contained 0.5 µl cDNA (~60 ng), 50% v/v SYBR Green PCR Master Mix, and 300 nM forward and reverse gene specific primers. Individual samples were loaded onto a 384-well optical PCR plate (catalog # MPS-3898, Phenix, Hayward, CA) using an Eppendorf epMotion 5070 robot (Eppendorf, Westbury, NY). Plates were then sealed with optical PCR sealing tape (catalog # LMT-OPCL, Phenix, Hayward, CA) and loaded onto an ABI 7900HT real-time PCR machine (Applied Biosystems, Foster City, CA) for thermal cycling (50ºC for 2 min, 95ºC for 10 min, 40 cycles at 95ºC for 15 sec and 60ºC for 1min) utilizing SDS 2.2 software (Applied Biosystems, Foster City, CA).

For initial primer testing, relative standard curves were generated from total RNA samples obtained from both horned and polled animals. RNA was serially diluted in 10-fold increments from 0.6 µg/µl to 0.00006 µg/µl, followed by reverse transcription. Adequate amplification efficiency within the linear range (C_t difference of -3.3 +/- 0.3) was verified and optimal cDNA concentration for analysis was identified.

Post-PCR dissociation curve analysis was used to verify presence of a single PCR product. Primers that did not meet these conditions were redesigned (*SYNJ1*) or were not used due to a lack of alternative primer sites (*SHH, CDRAP*). Additionally, no primers were designed for *SOX5* due to sequence assembly problems with the *SOX* gene family in bovine genome build 2.1 making it difficult to accurately identify the *SOX5* gene and its splice sites in bovine. The *TIAM1* gene was also not used in real-time RT-PCR because it was now placed outside of the polled critical interval and it has no known role in osteogenesis or chondrogenesis.

Differential expression of genes was compared by relative quantification of qRT-PCR data from horned, polled and scurred samples from male and female cattle. Three biological replicates and 3 technical replicates were performed on each plate, and *18S* rRNA and *GAPDH* were used for normalization. Other housekeeping genes were also tested, but *18S* and *GAPDH* exhibited the lowest standard deviation among these tissues in comparison to the others (*ACTB*, *YWHAZ*, and *SDHA*).

In cases where the gene expression was too low for accurate detection, reverse transcription of RNA samples was primed with gene-specific primers. For these genes (*C21orf45, C21orf62,* and *FOXL2*), the reverse primer was mixed in equal concentrations (0.25 μM each) with the reverse primers for *18S* and *GAPDH.*

Analysis of Real-Time PCR

Data were analyzed by relative quantification (Livak and Schmittgen, 2001). The geometric mean of the cycle threshold ($\mathbf{C_t}$) values for the *18S* and *GAPD* genes was

used as the endogenous control C_t for normalization, and a polled male (097T for 1 to 8 day or 751S for 5 to 6 mo) was used as the calibrator. Any obvious outliers (high standard deviation among technical replicates) were manually removed. Mean fold differences in expression among horned, polled, and scurred samples were calculated by averaging the relative quantity of each type. Additionally, mean fold differences in expression between male and female samples of each type were calculated.

Expression data were analyzed by the analysis of variance (ANOVA) procedure of SAS (SAS Institute Inc., Cary, NC). Factors investigated were sample type (horned, scurred, polled), sex and sex x sample type interaction. However, sex and sex x sample type interaction were not significant and were excluded from the final model. When an *F*-test was significant, Tukey's pairwise comparison ($\alpha = 0.05$) was used for means separation (Neter and Wasserman, 1974).

Results and Discussion

Qualitative Gene Expression

A total of 38 primers were designed to qualitatively examine gene expression profiles in various bovine tissues (Table A.5). β-Actin (***ACTB***) was included as a control. The primers for *SHH* were not specific, possibly due to the high sequence similarity among hedgehog gene family members. Analysis of expression of *SOD1* was complicated by the presence of an apparent processed pseudogene with ~97% similarity to the coding sequence of *SOD1* that appears to be novel to bovine and is currently placed on BTA13 (based upon bovine genome build 3.1).

All tissues tested expressed *ACTB*. Of the 37 other genes tested, 18 were expressed in the neonatal horn buds, 20 in polled skin, and 18 in both the neonatal horn buds and polled skin (Table 4.1). Five genes from the polled interval (*SFRS15, C21orf59, SYNJ1, C21orf66,* and *IL10RB*) were expressed in both horn buds and polled skin. In addition, *TIAM1* was expressed in both of these tissues. The gene *C21orf45* was weakly expressed in a single polled sample and *C21orf62* was relatively strongly expressed in a single polled sample, but these data were not reproduced. Additionally, 24 of the tested genes were expressed in the heart, 27 in the liver, 15 in the lung, 23 in the spleen, 20 in the muscle, and 29 in the brain. Three genes (*LOC440778*, *AGC1*, and *MAPK1*) were not expressed in any of the tissues tested. While there were some discrepancies between the duplicate tests, this could generally be attributed to low levels of gene expression within that tissue resulting in a weak band in one case and no visible band in the other, or human error.

Table 4.1. Qualitative assessment of gene expression in bovine tissues[1,2]

	Horn Buds				Polled Skin				Heart		Liver		Lung		Spleen		Muscle		Brain	
Gene	**282**	**282**	**598**	**598**	**10**	**10**	**268**	**268**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
TIAM1	-	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+
LOC150051	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
SFRS15	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
HUNK	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+
C21orf45	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
MRAP	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
C21orf63	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	-	-	+	-
C21orf59	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SYNJ1	+	-	+	-	-	-	-	+	+	-	+	-	-	-	+	+	+	-	+	+
C21orf66	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
C21orf62	-	-	-	-	-	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+
LOC440778	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OLIG2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
OLIG1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
IFNAR2	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
IL10RB	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IFNAR1	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+	-	+	-	+
SOX9	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+
COL2A1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
COL9A2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
AGC1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDRAP	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+
SOX5	-	+	-	-	-	+	-	+	-	+	+	+	-	-	+	+	-	+	+	+
SOX6	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+
PTHrP	-	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 4.1. Continued

	Horn Buds				Polled Skin				Heart		Liver		Lung		Spleen		Muscle		Brain	
	282	282	598	598	10	10	268	268	1	2	1	2	1	2	1	2	1	2	1	2
IHH	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
PRKCA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FGFR3	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+
STAT1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MAPK1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TWIST1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TWIST2	+	+	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	+	-	-
RUNX1	-	+	-	+	-	+	-	-	-	-	-	+	-	-	-	+	-	+	-	+
RUNX2	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+
COL18A1	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+
BMP4	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+
PISRT1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ACTB	+		+		+		+		+		+		+		+		+		+	

[1]+ = amplification product detected, - = no amplification product observed

[2]The numbers 10, 268, 282, and 598 represent the sample number of the polled skin or horn bud sample, respectively. The numbers 1 and 2 represent results from each of the duplicate PCR experiments.

The genes *SOX9*, *COL2A1*, *COL9A2*, *AGC1*, *SOX5*, and *SOX6* are known to play a role in bone development through cartilage formation (Lefebvre et al., 2001; Goldring et al., 2006). Expression of *COL2A1*, *COL9A2*, *AGC1*, or *SOX6* was not observed in mRNA from the neonatal horn buds suggesting that horn may form through intramembranous ossification rather than endochondral ossification via a cartilage intermediate. There was expression of *SOX5* and *SOX9*, but these cartilage markers are known to be expressed in skin and hair as well (Visel et al., 2004; Vidal et al, 2005). Genes such as *FGFR3* (Ornitz and Marie, 2002), *RUNX1* (Smith et al., 2005), *RUNX2* (de Crombrugghe et al., 2001), *TWIST1* (Yousfi et al., 2001), *TWIST2* (Yousfi et al., 2001), *STAT1* (Kim et al., 2003), *IHH* (Abzhanov et al., 2007; Kronenberg, 2003), *PTHRP* (Kartsogiannis et al., 1997), *BMP4* (Abzhanov et al., 2007; Pizette and Niswander, 2001), *PRKCA* (Oh et al., 2001; Radeff et al., 2004) and *MAPK* (Li et al., 2007) are known to have roles in both endochondral and intramembranous ossification or their developmental pathways. The presence or absence of expression of these genes in neonatal horn bud mRNA does not demonstrate whether the bone formation occurs by endochondral or intramembranous ossification, but it does provide clues into the regulatory network of early horn development.

Real-Time Reverse Transcriptase PCR

No significant ($P > 0.1$) differences in the levels of expression of genes from the polled interval were detected in horned and polled samples collected at 1 to 8 d of age. However, significant differences in expression between horned and polled samples were detected for *RUNX2* ($P = 0.01$), *FOXL2* ($P = 0.09$), *PRKCA* ($P = 0.02$), and *SOX9* ($P = 0.02$) (Figure 4.1). Expression of *RUNX2* was higher in horned samples than polled samples, whereas *SOX9*, *PRKCA* and *FOXL2* were expressed at higher levels in polled samples than horned samples. Expression of *BMP4* was higher ($P = 0.02$) in scurs than horns, while *C21orf62* was apparently expressed at higher levels ($P = 0.04$) in scurs than in polled skin. However, expression of this gene was generally very low and even when reverse transcription was driven by a gene specific primer a $C_t > 30$ was obtained, suggesting that quantitation for this gene is unreliable.

RNA quality from the 5 to 6 mo old polled samples was consistently better than that of the horned and scurred samples, probably a result of increased technical difficulty in extracting RNA from horn and scur tissue. The qRT-PCR results were normalized to

18S and *GAPD* to remove variation due to differences in RNA quality. Expression of *PRKCA* was higher ($P = 0.01$) in polled skin than in horns (Figure 4.2). Both *BMP4* ($P < 0.0001$) and *SYNJ1* ($P = 0.03$) were expressed at higher levels in polled skin than in either scurs or horns. Conversely, *PTHLH* ($P = 0.03$) was expressed at higher levels in polled skin and scurs than in horns. Finally, expression of *TWIST1* ($P = 0.05$) was higher in polled skin than in scurs while *C21orf66* ($P = 0.06$) tended to be expressed at higher levels in horns than in scurs.

In both the 1 to 8 d and 5 to 6 mo old samples, sex did not contribute to differences in expression across the 3 tissue types ($P > 0.1$). In the 1 to 8 d samples, there was a trend for *C21orf66* to be expressed lower in scurs from males than females, while *STAT1*, *TWIST1* and *FOXL2* tended to be expressed at higher levels in horns from males than females, and *C21orf62* tended to be expressed higher in polled skin from females than males (Figure 4.3).

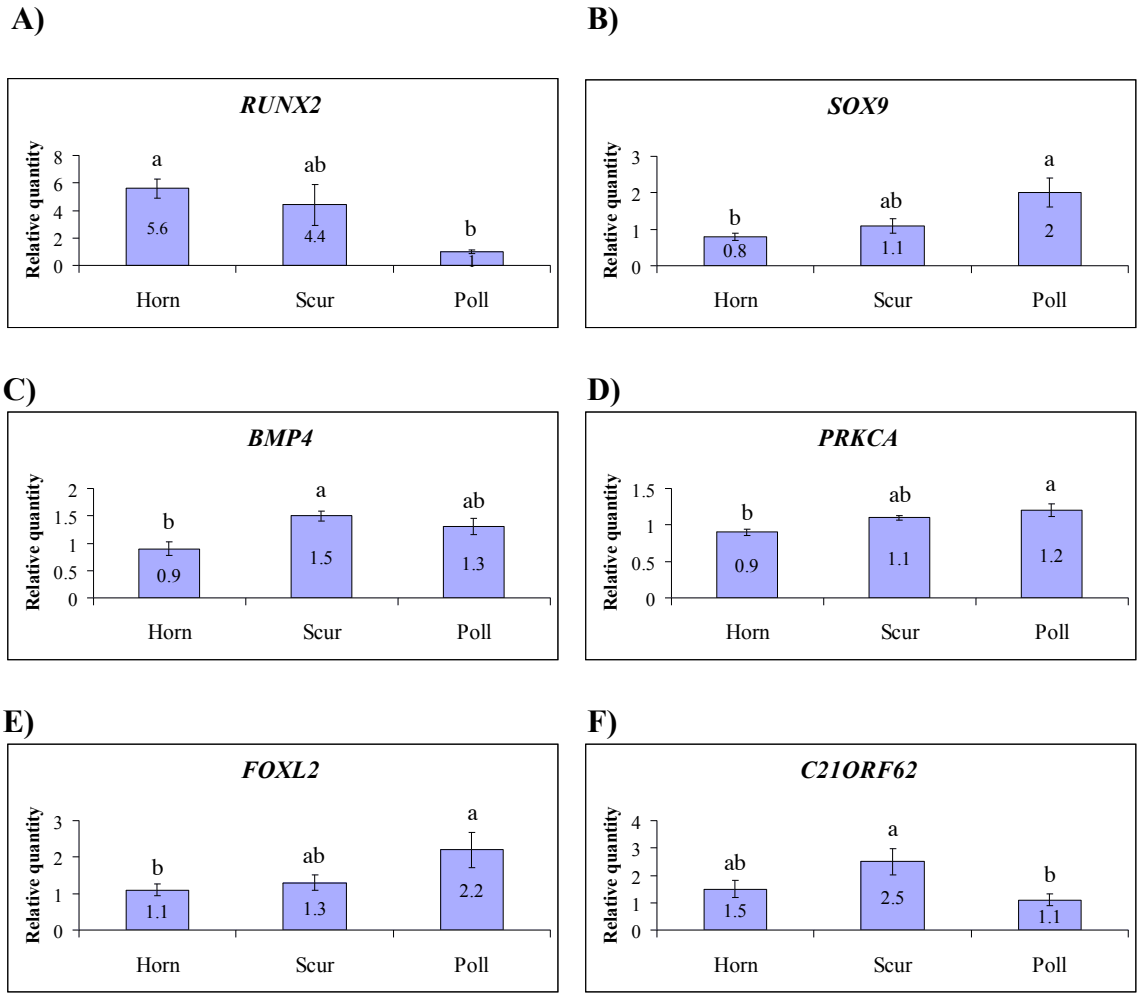


Figure 4.1. Means (relative quantity) and SE for expression in samples from 1 to 8 d old calves. The mean is given on each bar. Means with no letter in common differ ($P < 0.05$).

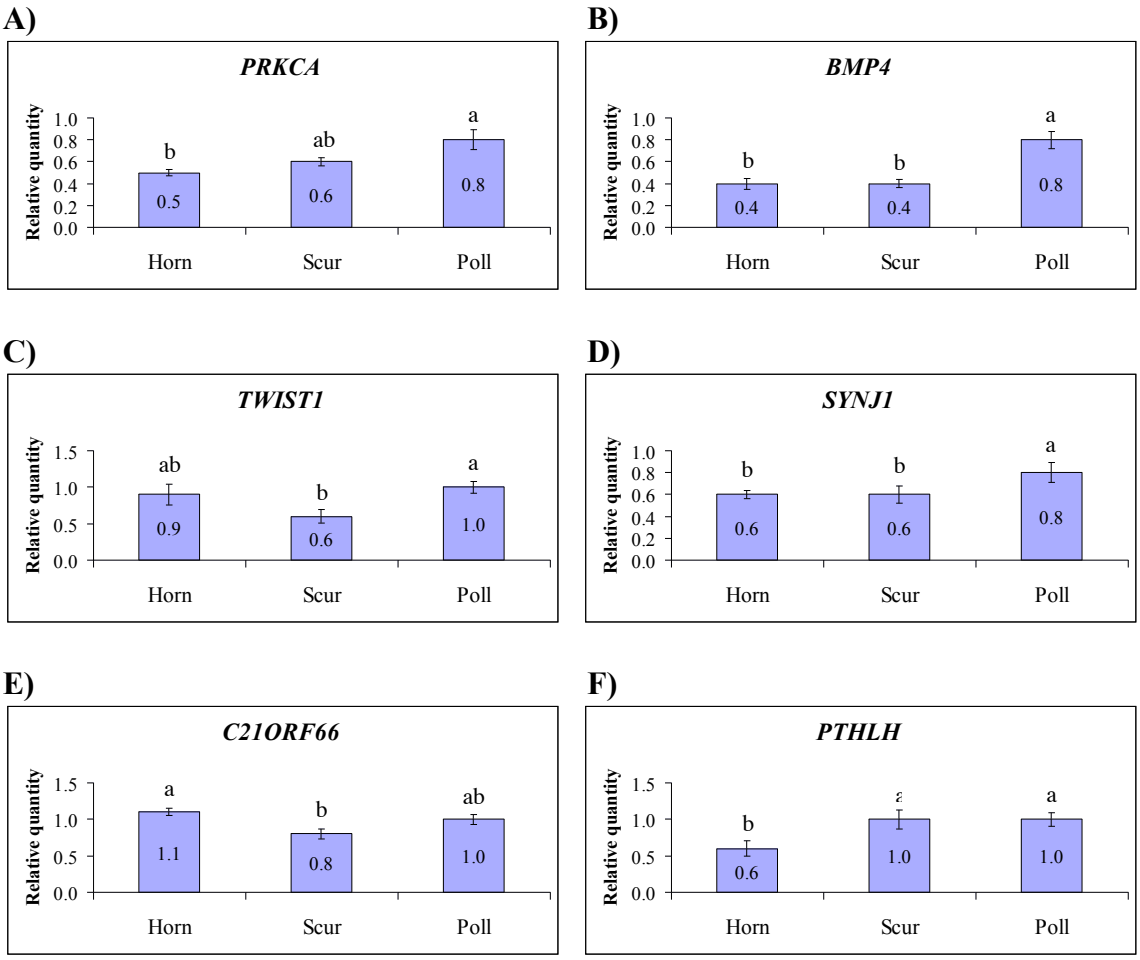


Figure 4.2. Means (relative quantity) and SE for expression in samples from 5 to 6 mo old calves. The mean is given on each bar. Means with no letter in common differ ($P < 0.1$).

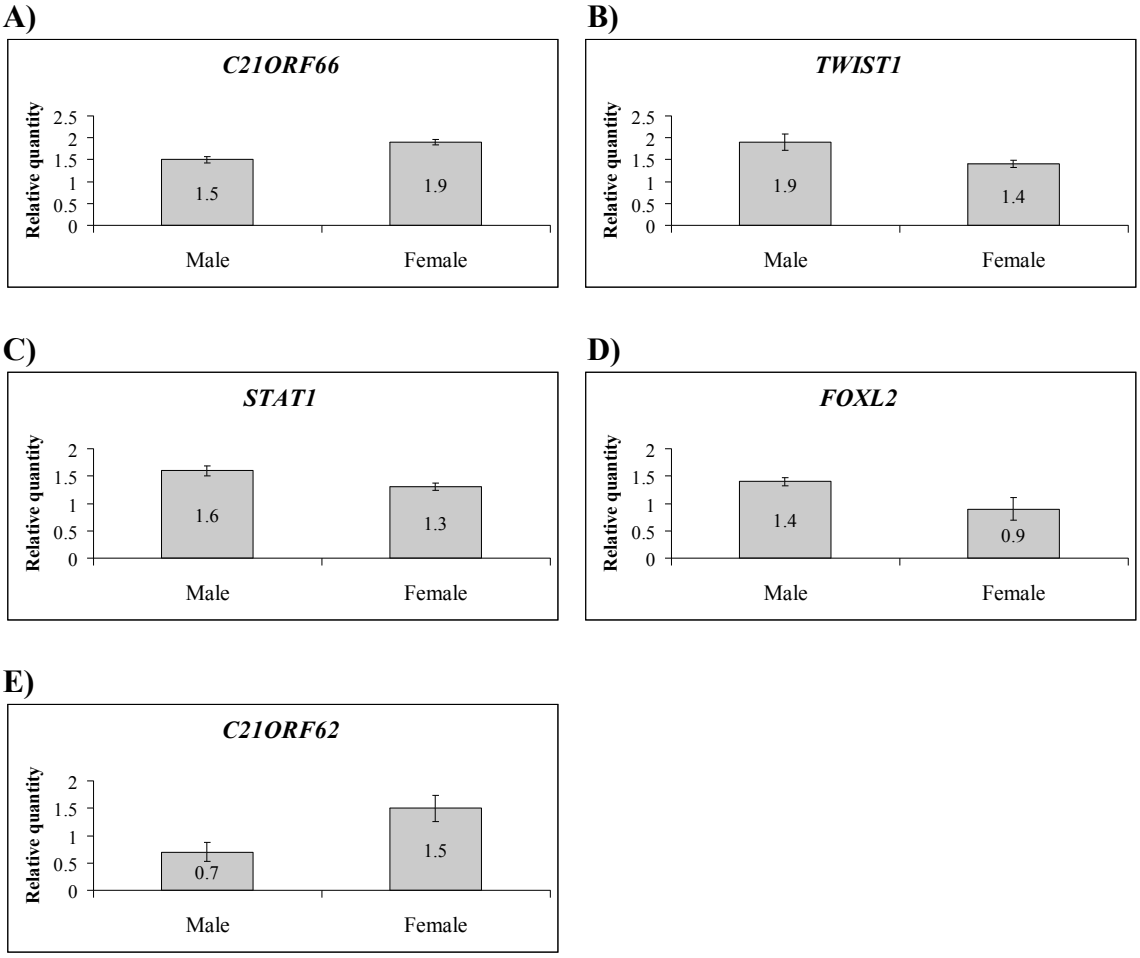


Figure 4.3. Means (relative quantity) and SE for expression in samples from 1 to 8 d old calves by sex that differed ($P < 0.1$). The mean is given on each bar. (A) scur (B-D) horn, (E) polled skin.

Interpretation

Two genes (*RUNX2* and *SOX9*) that are key regulators of bone development (Bi et al., 1999; Karsenty et al., 2001b) were differentially expressed in horn and polled skin samples from 1 d old calves. The RUNX2 gene is actively expressed within the osteoblasts that are required to produce new bone (Nakashima and de Crombrugghe, 2003). There was ~5-fold higher expression of *RUNX2* in horns than in polled skin at 1 d of age. By 5 to 6 mo, there was no difference between levels of expression of *RUNX2* in horns or polled skin. These data suggest that perhaps *RUNX2* is required to initiate bone formation in horns, but only a maintenance level, similar to that seen in the polled animals, is required during horn growth.

Expression of *SOX9* was ~2 fold higher in 1 d old polled samples than in scurs and horns suggesting that *SOX9* may act as an inhibitor of horn growth. It is known to down-regulate RUNX2 (Eames and Helms, 2004) and is generally known as an endochondral marker that is required for chondrogenesis (Bi et al., 1999; Goldring et al., 2006). However, *SOX9* is expressed by all early osteochondral progenitors, including those giving rise to osteoblasts (Ng et al., 1997; Zhao et al., 2002) and is also known to be expressed within the hair follicle (Vidal et al., 2005). Because differential expression of *SOX9* was not observed at 5 to 6 mo, the most likely interpretation of these results is that the expression differences seen in 1 to 8 d old calves is due to the expression of *SOX9* by early osteochondral progenitors.

Expression of *PRKCA* was higher in polled skin than horn at both 1 to 8 d and 5 to 6 mo. The PRKCA gene is part of the protein kinase C multigene family that

regulates chondrogenesis of mesenchymal cells through MAPK signaling (Oh et al., 2001). It is also involved in the proliferation of osteoblasts (Lampasso et al., 2002). Protein kinase C (particularly PRKCA) may have a role in bone remodeling through PTH signaling by stimulating the promoter activity of interleukin 6 resulting in bone resorption through actions on osteoclast precursors (Radeff et al., 2004). Because this gene was expressed at higher levels in polled skin than in horn, the role of PRKCA in developing horn is unclear and requires further investigation.

At ~1 d, expression of *BMP4* was higher in scurs and polled skin than in horns, while at 5 to 6 mo expression was higher in polled skin than in horns or scurs. Bone morphogenetic protein signaling is necessary for the formation of precartilaginous condensations, their differentiation into chondrocytes, and for maintenance of chondrogenesis. Previous studies suggested that *BMP* are master genes able to trigger the entire series of events needed for normal chondrogenesis (Pizette and Niswander, 2001). Signaling of BMP acts to direct cells towards a chondrogenic pathway at the expense of dermal (intramembranous) osteogenesis by inducing *SOX9* expression and inhibiting the expression of *RUNX2* and osteopontin (Abzhanov et al., 2007). However, Abzhanov et al. (2007) stated that BMP signaling is also important for intramembranous ossification because they are required for neural crest-derived mesenchyme to commit to the osteogenic pathway. For intramembranous ossification, bone morphogenic proteins (probably BMP2, BMP4, and BMP7) from the epidermis of the head are thought to instruct neural crest-derived mesenchymal cells to become bone cells directly (Gilbert, 1997). These BMP in turn activate RUNX2 in the mesenchymal cells. We expect that

expression of BMP4 would be higher in the embryo of horned calves and that levels diminished after activation of RUNX2. Onset of osteogenesis causing the scurred phenotype perhaps occurs later than horns explaining the higher levels of expression observed in this tissue at 1 d compared to horns and to 5 or 6 mo scurs. Other *BMP* family members such as *BMP2 and BMP7* should be investigated as well.

While *FOXL2* was differentially expressed, generally low levels of expression make these data somewhat unreliable. In goats, Pailhoux et al. (2001) showed a 3 to 4 fold excess of *FOXL2* in 70-dpc fetus horn buds of $PIS^{-/-}$ (polled) animals in comparison to the wild-type (horned). There is no known role for *FOXL2* in bone development, but it has been shown that SOX9 represses FOXL2 in the testis (Ottolenghi et al., 2007). Based upon this known interaction and the hypothesized involvement of *SOX9* in PIS (Pailhoux et al., 2001), it is possible that FOXL2 is involved in horn development through an interaction with SOX9.

Because there was not a significant difference in gene expression for any of the genes located within the polled interval, these results suggest that the polled locus most likely has its primary effect during fetal development. In addition, several of the genes with significant differences in expression are early regulators of bone development. An embryonic developmental time course to examine changes in expression of these genes is needed.

CHAPTER V

HORN HISTOLOGY AND IN SITU HYBRIDIZATION

Introduction

Throughout the 19th century, several studies examining horn structure were reported but the results were contradictory (Dove, 1935). Anatomists (e.g., Jaeger, 1839; Owen, 1868; Siedamgrotzky, 1870 cited by Dove, 1935) reported the horn to be an outgrowth of the frontal bone. However, several studies (e.g., Sandifort, 1829; Numan, 1848; Nitsche, 1898 cited by Dove, 1935) reported that the horn core originated from a separate center of ossification that subsequently fused to the frontal bone. Horn bud transplantation experiments demonstrated that this was indeed the case because tissues lying above the periosteum at the site of a future horn could be transplanted to other regions on the frontal and parietal bones and still result in the formation of a horn (Dove, 1935). Despite these data, several more recent studies still described the horn as an outgrowth of the frontal bone (Pasquini et al., 1982; Georges, 1993; Asai et al., 2004).

The mechanism of ossification of the horn core was also controversial. Several studies reported the presence of cartilage within the developing horn (e.g. Sandifort, 1829; Numan, 1848; Gadow, 1902 cited by Dove, 1935) suggestive of endochondral ossification. Conversely, as cited by Dove (1935), Lataste (1894) and Fambach (1901) reported that the horn core formed directly by dermal (intramembranous) ossification without cartilage.

Objectives

The objective of the study was to characterize the histology of developing horns and to localize patterns of gene expression to specific cell types by in situ hybridization. Identifying the type of bone development occurring within the horn as evidenced by histology and gene expression is key knowledge needed to understand the developmental pathway leading to horn development.

Materials and Methods

Horn Collection

Horns for histological studies were collected from animals raised at the Texas A&M University research station in McGregor. Horns were removed from 1 d old and 5 to 6 mo old calves as described in Chapter IV. Horns and scurs were also collected at the time of slaughter from approximately 1.5 yr old steers. These were removed from the skull using a saw, and dissected into halves using a band saw. Selected horns and scurs were placed into 4% paraformaldehyde for fixation.

Fixation

Horn samples were left in 4% paraformaldehyde for 12 to 48 hr (approximately 24 hr per 1 cm section). Samples were then rinsed with 50% ethanol, and left covered in 50% ethanol for 8 to 24 hr. Samples were subsequently covered for 8 to 24 hr each with 50% ethanol, 70% ethanol, and finally stored in 70% ethanol until further processing.

Histology

Embedding and staining for histology was performed by the Texas A&M Department of Veterinary Integrative Biosciences histology lab where tissues were decalcified for 24 hr in a 1:1 solution of 50% formic acid: 20% sodium citrate, rinsed with water, and again stored in 70% ethanol until processed further. Tissues were then dehydrated through a graded series of alcohols (70% ethanol for 2 hr, 85% ethanol for 2 hr, 2 repeats of 95% ethanol for 2 hr, and 2 repeats of absolute ethanol for 2 hr). Tissues were then cleared 3 times in xylene for 2 hr each, and infiltrated in paraffin for 3 hr. After processing, the tissue was embedded in paraffin and sections were cut to a thickness of 5 μm for routine histology and in situ hybridization. All polled animals were Angus, while horn and scur samples were from *Bos indicus* (Brahman and Nellore) influenced animals. Horned and polled samples from 1 d old calves (tag no. 282, 598, 269, 173), as well as horn samples from a 5 mo old (tag no. 787S), a 6 mo old (tag no. 411S), as well as horn and scur samples from 1.5 yr old steers (tag no. 8041, 9701, 7202, 7019, 8403, 7519, 7701, 8030, 7020, 8035, 8044) were stained for histological analysis using hematoxylin and eosin and coverslipped with permount.

Probe Construction

Amplicons of 300 to 500 bp in length were used to generate probes for ISH. Primers (Table A.15) were optimized using cDNA from tissues known to express the respective genes (Chapter IV). These PCR products were cloned into the PCR2 vector using the PCR2 dual promoter TA cloning kit (Invitrogen, Carlsbad, CA) according to

the manufacturer's instructions. Briefly, PCR products were mixed in a 1:1 molar ratio with the PCR2 vector, ligated together at 14ºC overnight, transformed into TOP10F' competent cells by heat shock at 42ºC, and plated on LB-agar plates containing 100 µg/ml ampicillin, 0.05 mg/ml IPTG, and 0.04 mg/ml X-gal and grown at 37ºC overnight. White colonies were then selected from this plate and used to inoculate 5 mL LB containing 100 µg/ml ampicillin, 0.05 mg/ml IPTG, and 0.04 mg/ml X-gal. These cultures were grown at 37ºC overnight with shaking at 250 rpm on a ThermoForma model 420 orbital shaker (Thermo Electron Corporation, Marietta, OH). Following a 16 to 20 hr incubation, 500 µl of culture was mixed with 500 µl 2X freezing solution (72 mM K_2HPO_4, 26 mM KH_2PO_4, 3.4 mM sodium citrate, 0.8 mM $MgSO_4$, 13.6 mM $(NH_4)_2SO_4$, 8.8% glycerol) to produce a glycerol stock that was stored at -80 ºC. A dilution from the glycerol stock was used directly in PCR with universal primers as described in Chapter III and amplicons were sequenced with PUC forward and reverse primers to determine the orientation of the insert.

Plasmid DNA was isolated using the Plasmid Maxi-kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Aliquots of plasmid DNA (~20 µg) were digested with 50 U *BamHI* (Promega, Madison, WI) and *EcoRV* (Promega, Madison, WI) at 37 ºC overnight. Digested DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform, then precipitated with 1/10 volume 2M NaCl and 2.5 volumes of absolute ethanol at -80ºC for 1 hour. Samples were centrifuged at 13000 *g* for 10 min, rinsed with 70% ethanol, air dried and then re-suspended in 50 µl sterile water.

Labeling with Digoxigenin

Probes were labeled with digoxigenin-UTP by in vitro transcription using the DIG RNA Labeling (SP6/T7) kit (Roche, Indianapolis, IN; cat. No. 11175025910) according to the manufacturer's instructions. The T7 promoter was used for *BamHI* digestions and the SP6 promoter for *EcoRV* digested products to obtain anti-sense and sense probes dependent on insert orientation. Probes were subsequently purified using Centri-Sep columns (Princeton Separations, Inc., Adelphia, NJ). Concentration of the probe was estimated using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Dot blots were used to determine the labeling efficiency of the probes as per the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). This was followed by immunological detection using the DIG Nucleic Acid Detection kit (Roche Applied Science, Indianopolis, IN; cat. no. 11175041910) according to the product manual, except volumes were reduced to 10 to 20 mL dependent on the size of the blot. Based upon these results, probes were diluted to a working concentration of 10 ng/µl.

In Situ Hybridization Using DIG-Labeled Probes

Slides were dewaxed using Citrisolv (Fisher, Atlanta, GA) and then rehydrated through a graded series of ethanol (100%, 95%, 70%) followed by two 2 min washes in DEPC-treated water. Slides were then rinsed twice in PBS for 5 min, permeablized in 10 µg/ml proteinase K for 10 min, washed twice in DEPC-PBS with 10 mM glycine for 3 min, acetylated twice in 0.1M triethanolamine buffer containing 0.25% acetic

anhydride for 5 min, rinsed 2x in DEPC-PBS for 5 min, and then placed into pre-hybridization buffer (50% formamide, 4X SSC). Sections were covered with 30 µl of hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1X Denhardt's solution, 4X SSC, 10 mM DTT, 1 mg/ml yeast t-RNA, 1 mg/ml sheared salmon sperm) containing 10 ng DIG-labeled RNA probe. These sections were then coverslipped, sealed with rubber cement, and placed into a humid chamber at 50ºC to 55ºC overnight. Slides were dipped into 2X SSC to remove the coverslip and then washed twice in 2X SSC for 15 min with agitation at 37ºC. Single-stranded RNA was then digested in NTE buffer (500 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA) containing 20 µg/ml RNaseA for 30 minutes, followed by 2 washes in 1X SSC for 15 min at 37ºC. Slides were then placed into 1% blocking solution (Roche Applied Science, Indianapolis, IN) for 30 min, covered in 1:500 antibody solution (anti-DIG AP conjugate in 1% blocking reagent), coverslipped, and allowed to incubate for 2 hr in a humid chamber. Coverslips were then removed and slides washed twice in buffer 1 (100mM Tris-HCl, 150 mM NaCl, pH 7.5) for 10 min, and then in detection buffer (0.1M Tris-HCl, 0.1M NaCl, 50 mM $MgCl_2$, pH 9.5) for 10 min. Sections were then covered in ~ 30 to 50 µl color solution (1 ml detection buffer, 20 µl Roche NBT/BCIP solution, 1 mM levamisole) per section, coverslipped, sealed with rubber cement, and placed into a humid chamber for ~2 days. Slides were then rinsed in buffer 3 (10mM Tris-HCl pH 8.1, 1 mM EDTA) for 5 min, and then twice in water for 2 min. Some sections were then mounted with Crystal/Mount (Biomedia Corp., Foster City, CA) while others were counterstained using Fast Green FCF (Mallinckrodt Baker, Inc., Phillipsburg, NJ). Counterstaining was

performed by immersing sections in a solution of 0.1% Fast Green, 1% acetic acid for 10 sec to 2 min. Slides were rinsed 3 times in water for 2 min and dehydrated through a series of graded ethanol (2 repeats of 70%, 95%, 100% for 2 min each). Slides were then placed into citrisolv (Fisher, Atlanta, GA) until they were coverslipped using permount (Fisher, Atlanta, GA). Positive control probes for *18S* or *RUNX2* or both, as well as a negative (no probe) control were included in each run. A technical replicate at a later date was performed for each gene.

Results and Discussion

Microscopic Observations of Hematoxylin and Eosin Stained Sections

The core of the horn of *Bos indicus* influenced calves was composed of trabecular bone that did not appear to have a cartilage intermediate. Chondroblasts, chondrocytes, or cartilage were not observed in any of the 1 d or 5 to 6 mo samples. Patches of cartilage were observed in 1.5 yr old horn samples but only peripherally.

Within the 1 d old samples, bone formation in the developing horn was in its earliest stages. A layer of muscle and connective tissue with blood vessels, veins and nerves was present above the trabecular bone (Figure 5.1). The epidermis of the horned samples was different from that of polled samples largely due to an increased size of the stratum basal and onset of keratinization in what will become the sheath of the young horns in comparison to the polled skin.

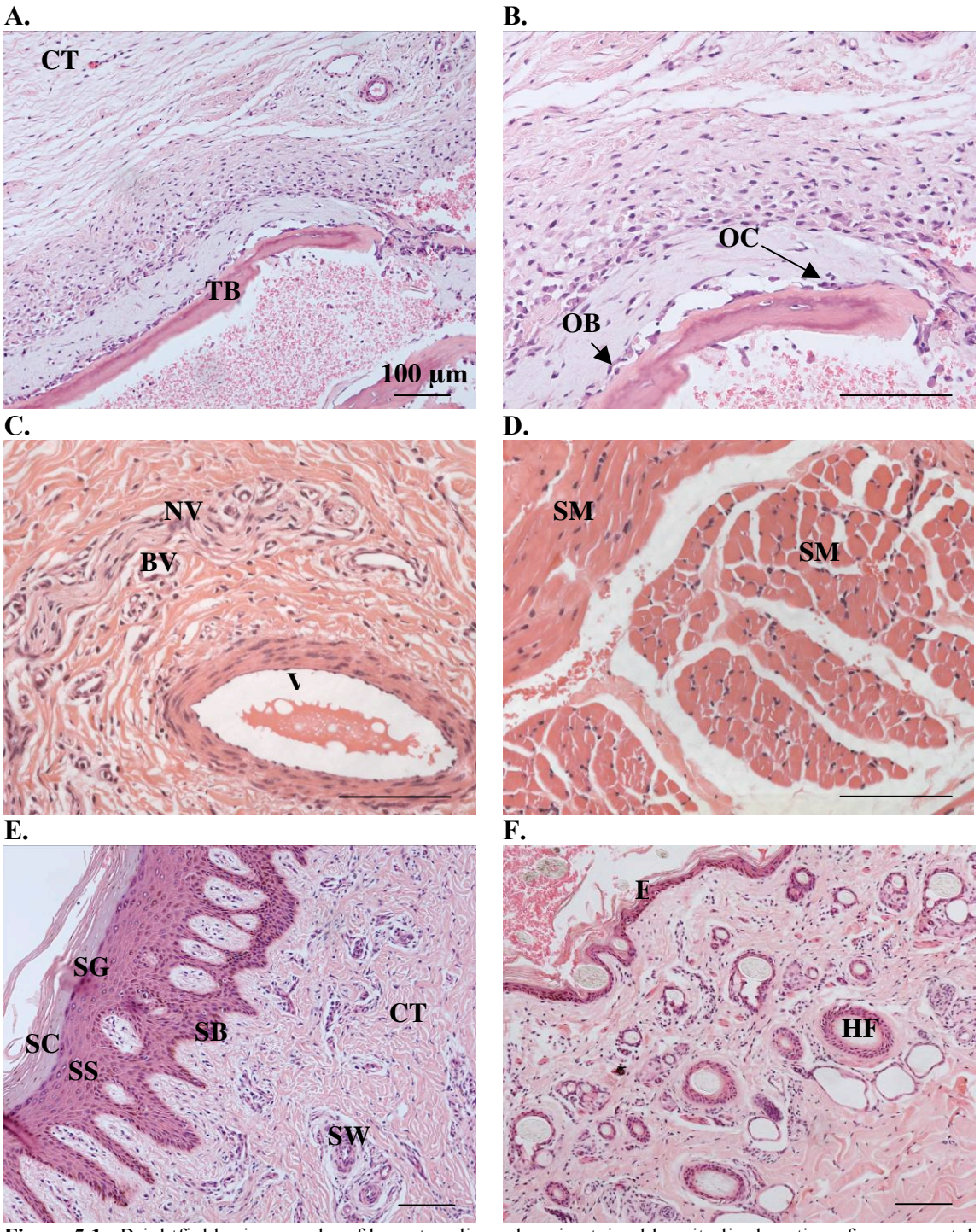


Figure 5.1. Brightfield micrographs of hematoxylin and eosin stained longitudinal sections from neonatal horn buds and polled skin. **CT** = connective tissue, **TB** = trabecular bone, **OC** = osteoclast, **OB** = osteoblast, **NV** = nerve, **BV** = blood vessel, **V** = vein, **SM** = skeletal muscle, **SC** = stratum corneum, **SG** = stratum granulosum, **SS** = stratum spinosum, **SB** = stratum basal, **SW** = sweat gland, **HF** = hair follicle, **E** = epidermis. A) Horn Bud, animal 598. B) Horn Bud, animal 598. C) Horn bud, animal 598. D) Polled skin, animal 268. E) Horn Bud, animal 598. F) Polled skin, animal 268.

In the 5 and 6 mo old horn samples, the bone has begun to form a dome like structure protruding outward from the skull. All bone observed within the horn was trabecular bone, and this was surrounded by newly forming bone still lacking a cartilage intermediate (Figure 5.2). There was a layer of mesenchymal connective tissue surrounding the bone, and this was surrounded by a layer of collagenous connective tissue. Nerves, blood vessels, and veins were present within the connective tissue, and there was an artery in the 6 mo old horn section.

In the 1.5 yr old horn samples (Figure 5.3), there was active bone growth at the edges of the trabecular bone as demonstrated by osteoblastic activity. The trabecular bone was still surrounded by mesenchymal connective tissue, but the bony core had increased vastly in size in comparison to the 5 and 6 mo old horns. The blood vessels within these sections were large and the layer of connective tissue between the horn and the skin had become much smaller.

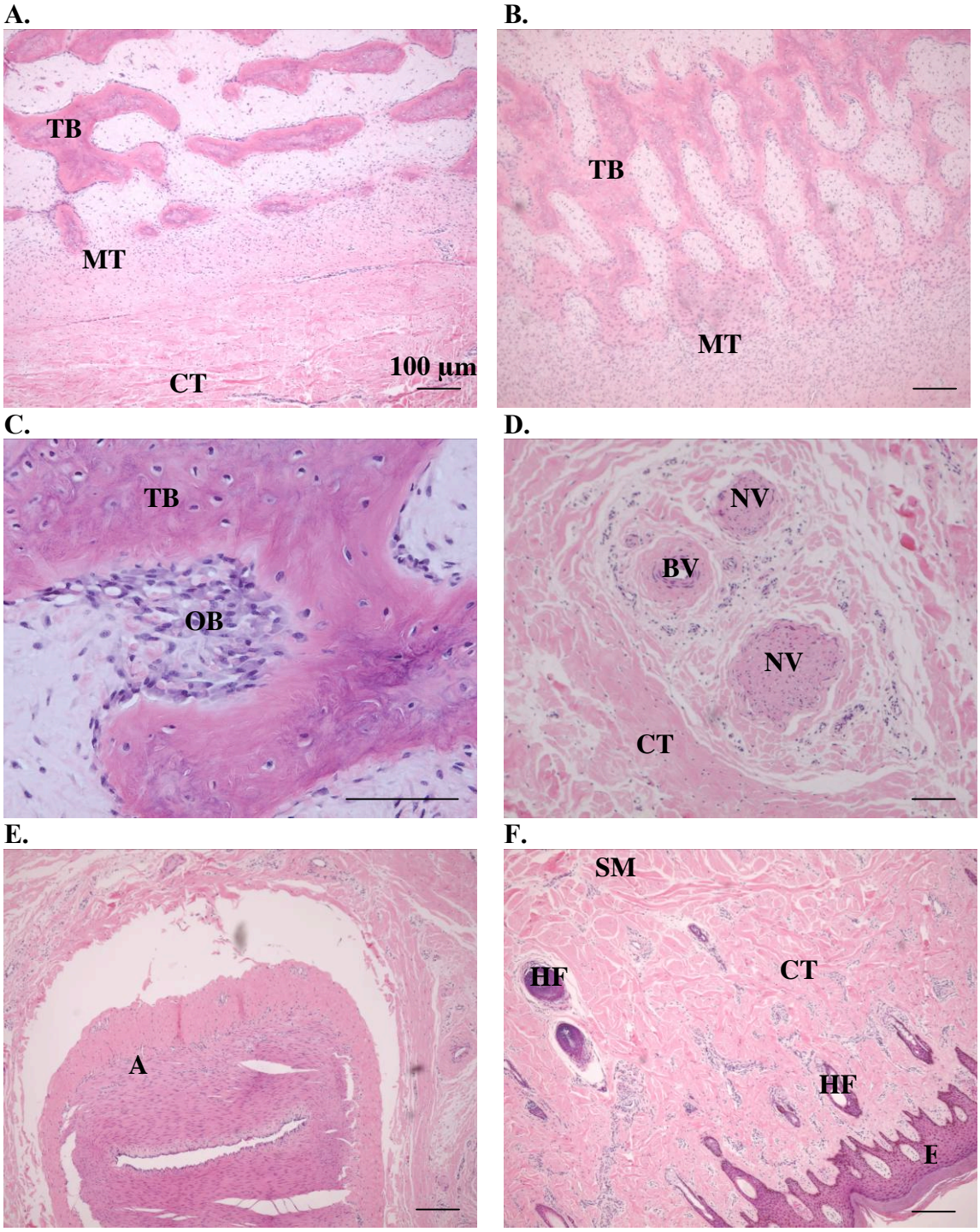


Figure 5.2. Brightfield micrographs of hematoxylin and eosin stained longitudinal sections from 5 mo (787S) and 6 mo (411S) horn. **TB** = trabecular bone, **MT** = mesenchymal connective tissue, **CT** = collagenous connective tissue, **OB** = osteoblast, **NV** = nerve, **BV** = blood vessel, **A** = artery, **SM** = skeletal muscle, **HF** = hair follicle, **E** = epidermis. A) 787S. B) 411S. C) 787S. D) 411S. E) 411S. F) 411S.

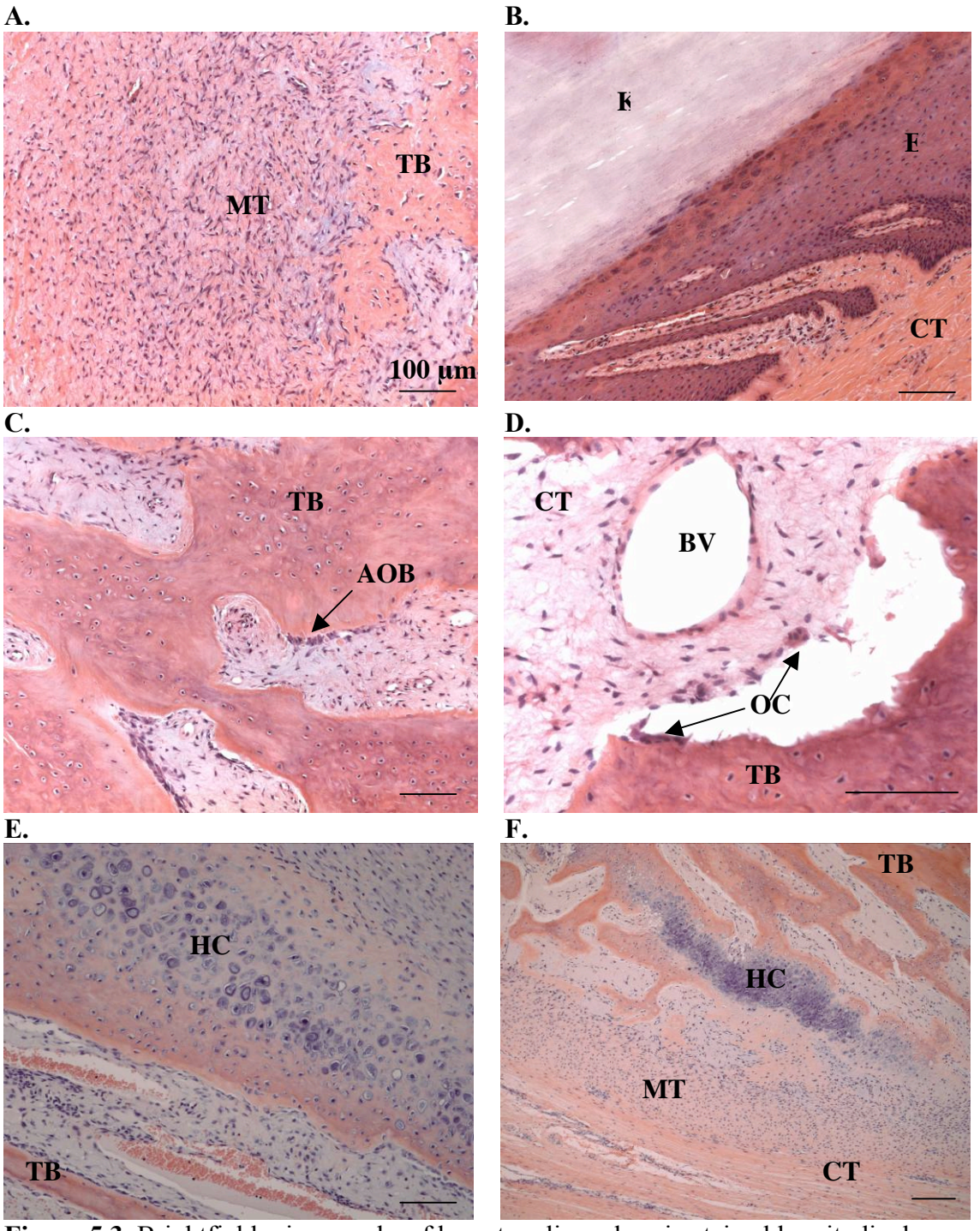


Figure 5.3. Brightfield micrographs of hematoxylin and eosin stained longitudinal sections from ~ 18 mo horn. **MT** = mesenchymal connective tissue, **TB** = trabecular bone**, K** = keratin, **E** = epidermis, **CT** = connective tissue, **AOB**= active osteoblasts, **BV** = blood vessel, **OC** = osteoclast, **HC** = hyaline cartilage. A) 9701. B) 9701. C) 9701. D) 9701. E) 8030. F) 7020.

Some ~1.5 yr old scurred samples were stained for histological analysis as well. While there was considerable variation among the scur samples collected at slaughter, there appeared to be 2 distinct types of scurs (Figure 5.4). The majority of these were small, loose growths. There was generally a small protrubence of the frontal bone, but the rest of the scur consisted of collagenous connective tissue covered by a keratin sheath. The second form of scurs consisted of an obviously separate bone within the connective tissue. Upon histological examination of some of these (Figure 5.5), patches of ectopic hyaline cartilage were observed. It is interesting to note that in at least two cases ectopic hyaline cartilage was observed on the frontal boss that is known to originate as intramembranous bone. As discussed by Day et al. (2005), the genetic inactivation of *β-catenin* causes ectopic formation of chondrocytes, so this observation is not unprecedented. Chondrocytes and osteoblasts differentiate from common mesenchymal progenitors. There appears to be competition or mutual suppression between the genetic pathways that lead to either osteoblast or chondrocyte differentiation in the common mesenchymal progenitors during both endochondral and intramembranous ossification. If a key gene is inactivated (e.g. *SOX9, OSX, CTNNB1*), the mechanism of osteoblast differentiation can switch to chondrocyte differentiation, or vice versa.

A)

B)

Figure 5.4. Examples of the 2 types of growths (scurs) seen in animals heterozygous at the polled locus (Pp). A) Small scurs that do not contain a separate center of ossification. B) Scur consisting of 2 separate bone growths.

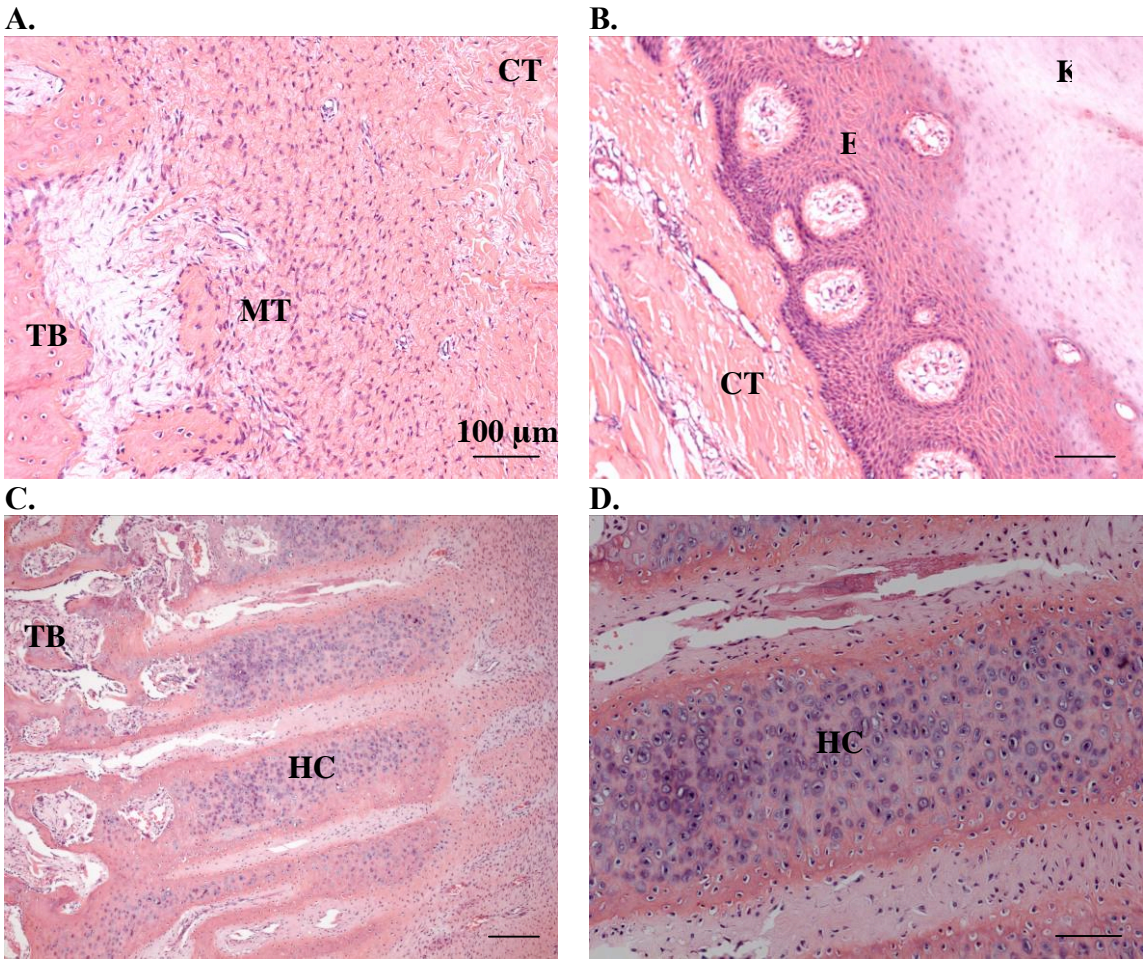


Figure 5.5. Brightfield micrographs of hematoxylin and eosin stained sections from ~ 18 mo scurs. Macroscopically, animal 8403 had small scurs consisting of frontal boss, connective tissue layer, and keratin sheath while 7701 had scurs consisting of a frontal boss and a completely separate bony core surrounded by connective tissue and covered in a keratin sheath. **TB** = trabecular bone, **MT** = mesenchymal connective tissue, **CT** = connective tissue**, E** = epidermis, **K** = keratin, **HC** = hyaline cartilage, **OB** = osteoblasts. A) 8403. B) 8403. C) 7701. D) 7701.

The results of the current study suggest that the normal mode of horn development in *Bos indicus* influenced cattle is through an intramembranous ossification, although ectopic cartilage can sometimes be observed. This may explain the contradictory observations in many previous studies. It is interesting to speculate that ectopic cartilage within some horns might be an expression of the scur phenotype.

In Situ Hybridization

In situ hybridization with a radioactive or non-radioactive label was attempted for several genes (*SFRS15*, *SYNJ1*, *IL10RB*, *C21orf59, TIAM1* and *TWIST1*) but the levels of expression were apparently lower than the detection limit of this method. Due to the high GC content and extensive regions of similarity to other members of the FOX gene family, it was not possible to design a robust probe for *FOXL2*. Expression of *C21orf66* was tentatively localized to the epithelial lining of the blood vessel, the osteoblasts, and the hair follicle using a radioactively labeled probe, but replicate experiments were not consistent.

Expression of *RUNX2* and *SOX9* was localized to particular cell types by in situ hybridization. Expression of *RUNX2* was detected in osteoblasts, and weaker expression was observed in the sebaceous glands (Figures 5.6 and 5.7). These results were not

unexpected because RUNX2 is a transcription factor that is known to control osteoblast differentiation (Karsenty et al., 2001b). Furthermore, sebaceous or cutaneous horns are the horn-like growths in human that can result from hard outgrowths of the contents of a sebaceous cyst (Arvas et al., 2007). Expression of *SOX9* was also localized to the sebaceous gland (Figure 5.8), which is consistent with Chen et al. (2006). While *SOX9* is also known to be expressed in chondrocytes and osteochondral progenitors (Ng et al., 1997; Zhao et al., 2002), these cells were not observed in the horned or polled sections.

As described by Day et al. (2005), down-regulation of *SOX9* and upregulation of *RUNX2* (Chapter IV) suggests an intramembranous mode of bone development, which is consistent with our histological observations. Based on these combined data, we propose that the polled locus is upstream of RUNX2 and SOX9 in the osteogenic pathway, and could have its primary effect on the differentiation of mesenchymal condensations.

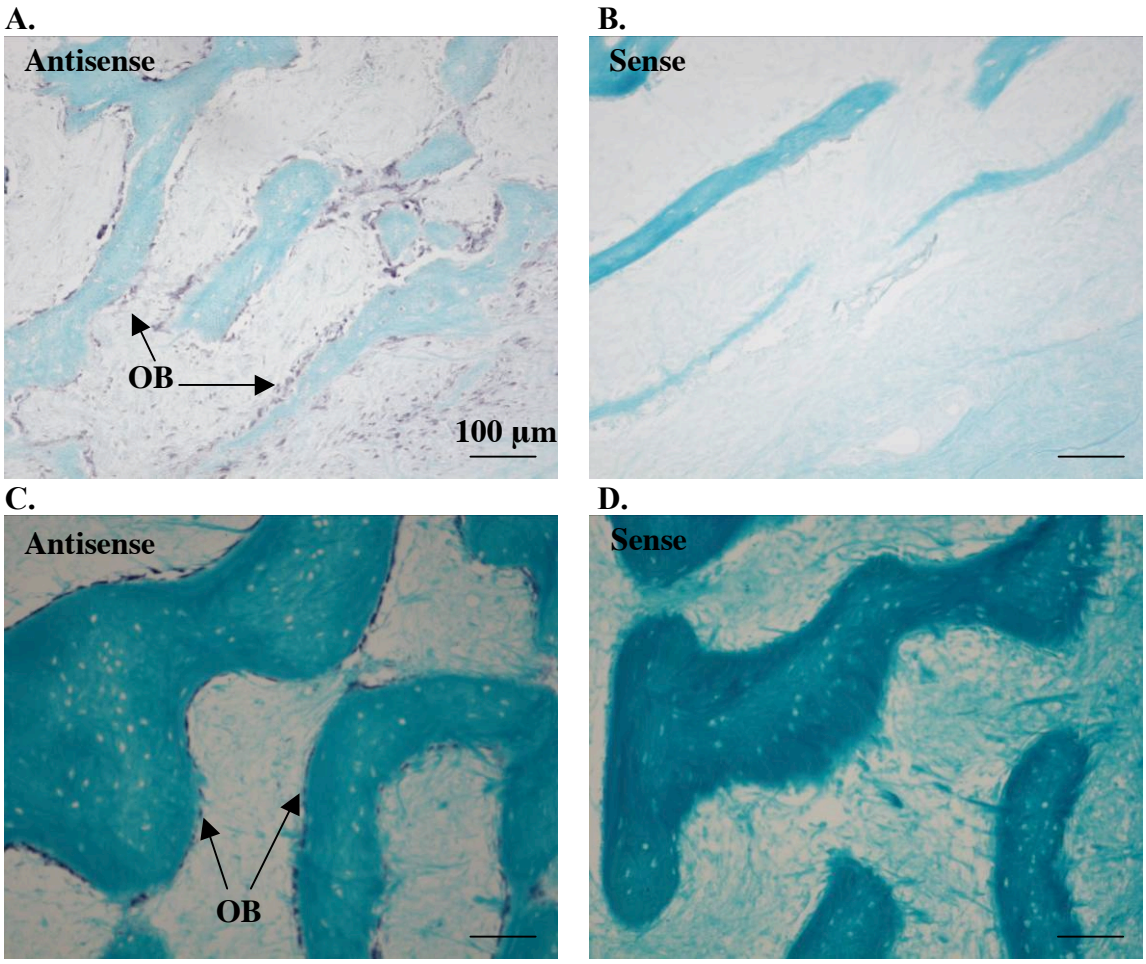


Figure 5.6. Brightfield micrographs demonstrating *RUNX2* expression within osteoblasts. Slides counterstained with 0.1% fast green, 1% acetic acid. **OB** = osteoblasts. A-B) Neonatal horn buds. C-D) 5 mo old horns.

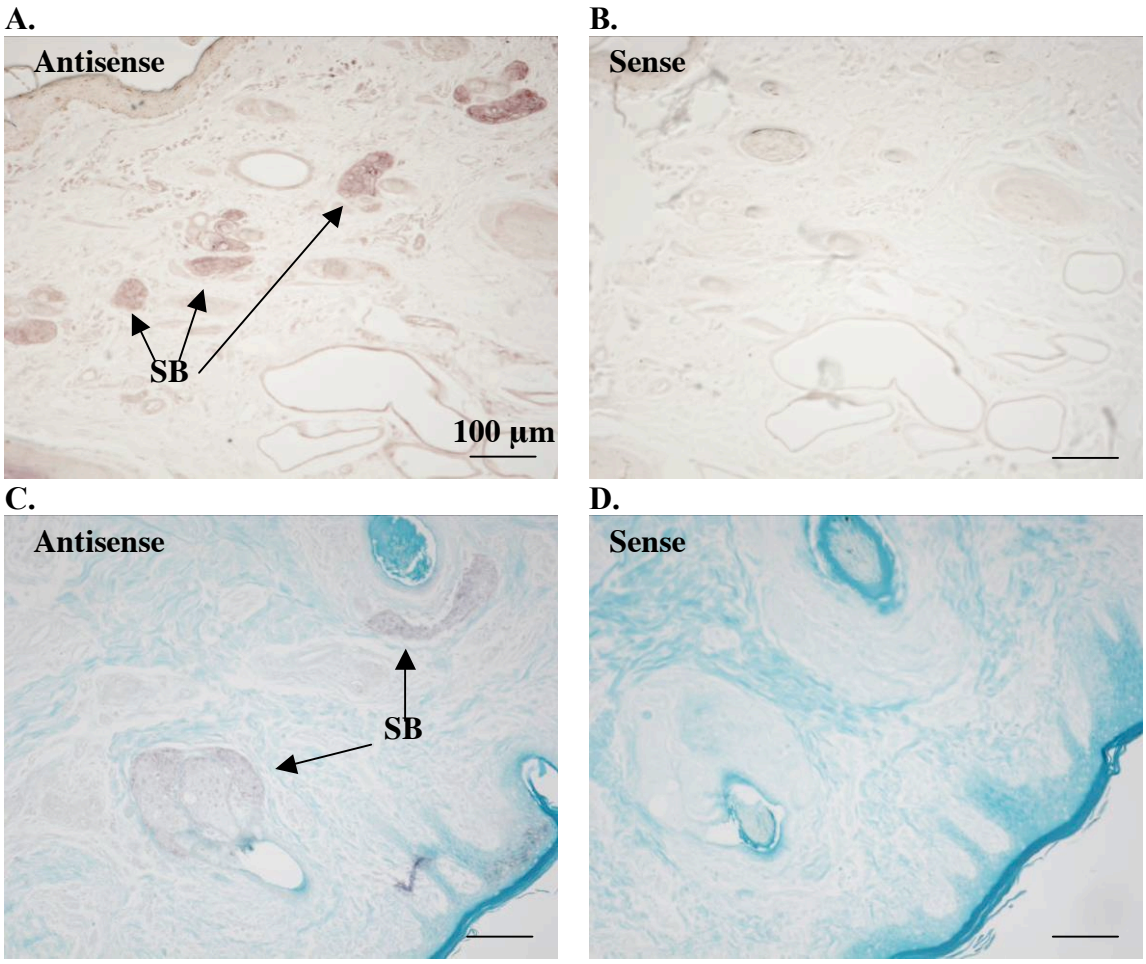


Figure 5.7. Brightfield micrographs demonstrating *RUNX2* expression within the sebaceous gland. **SB** = sebaceous gland. A-B) Neonatal polled skin, not counterstained. C-D) Neonatal horn, counterstained with 0.1% fast green, 1% acetic acid.

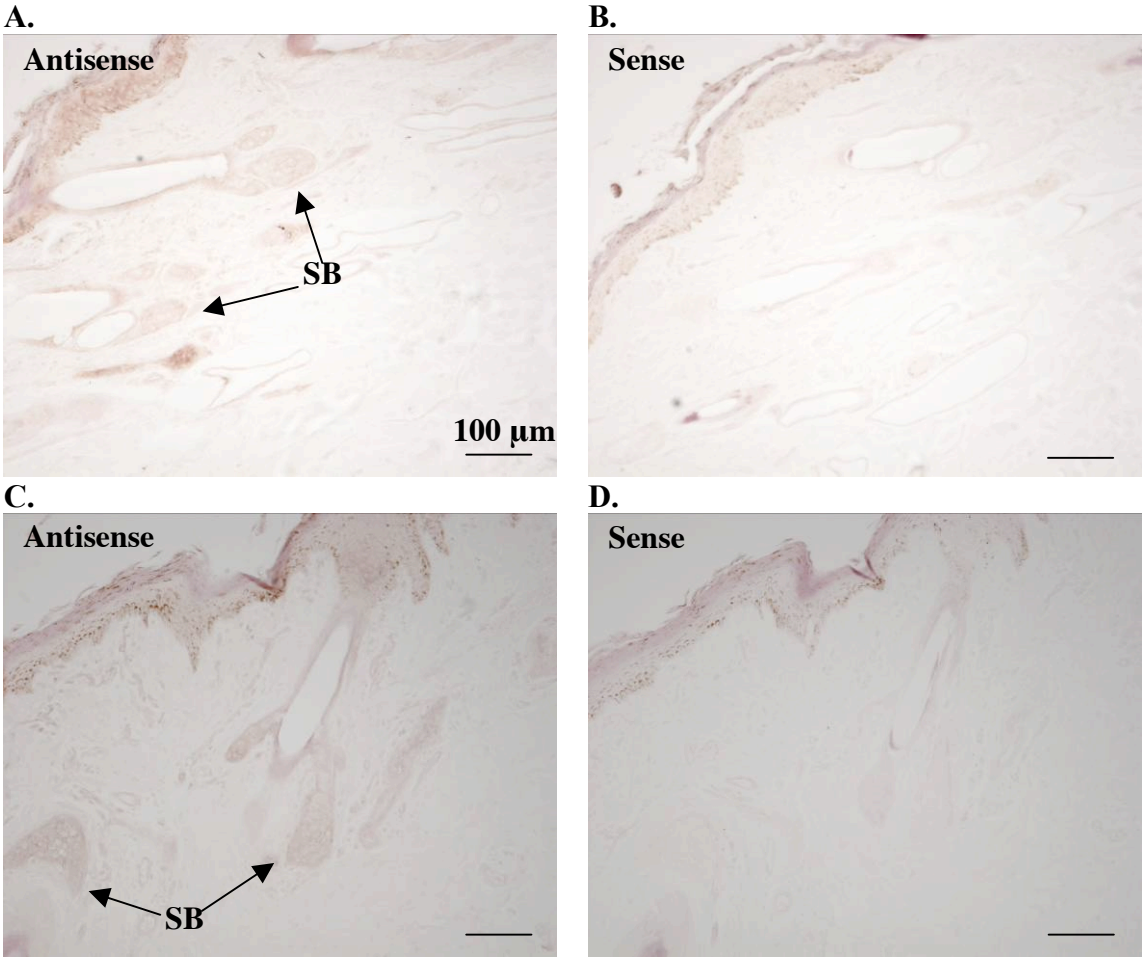


Figure 5.8. Brightfield micrographs demonstrating *SOX9* expression through ISH. **SB** = sebaceous gland. A-B) neonatal polled skin, not counterstained. C-D) neonatal horn, not counterstained.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Polled is an economically important trait in cattle, but the gene causing this phenotype has yet to be discovered. The objective of this research was to characterize the genomic region containing the polled locus (*IFNAR1* to *SOD1* on BTA1) and to functionally characterize genes from the interval plus other genes with known roles in bone development. These data have not only moved us closer toward positionally cloning this gene, but have also added to our understanding of the biology of horn development.

A 2.5 Mb BAC contig was constructed from Angus, Longhorn and horned Hereford DNA that encompasses the region identified by Song (1998) and Stillwell (1998), as well as that of Drögemüller et al. (2005). This contig did not reveal any rearrangements in gene order compared to HSA21, but did reveal problems associated with the order of scaffolds within the bovine genome (build 2.1). This contig allowed the accurate positioning of genes within the contig.

Our hypothesis was that the polled locus is a tissue specific transcription factor that is expressed in the developing horn buds and acts directly or indirectly upon SOX9. Therefore, *C21orf66* which was the only transcription factor from the interval to be expressed in horn and skin at 1 d of age, was sequenced along with its 5' and 3' ends. While no single SNP was identified that could be attributed as the causative mutation for polled, 144 SNP were discovered among 7 horned and polled cattle breeds. These SNP

are a valuable resource for future linkage disequilibrium and association analyses. In addition, the structure of the bovine *C21orf66* gene was investigated. It contains 18 exons and spans 30,976 bp of genomic DNA. A putative alternative transcript for this gene was also described.

Gene expression was evaluated by both qualitative and quantitative methods. Five genes from the polled interval and 13 genes with known roles in chondrogenesis and osteogenesis were expressed in skin or horn. None of the genes from the polled interval were differentially expressed in skin and horn from 1 d old *Bos indicus* influenced calves. However, there were significant differences in the levels of expression of *RUNX2*, *SOX9*, *BMP4*, *PRKCA*, and *FOXL2* in these samples. At 5 to 6 mo of age, *PRKCA, BMP4, SYNJ1,* and *PTHLH* were differentially expressed in horns and polled skin. Expression of *RUNX2* was localized to the osteoblasts and both *RUNX2* and *SOX9* were expressed in sebaceous glands of horn sections from 1 d old *Bos indicus* influenced calves.

The combination of results from analysis of gene expression and histological examination of horns and scurs from newborn, 5 to 6 mo, and ~1.5 yr old *Bos indicus* influenced cattle suggest that horns form through intramembranous ossification. No cartilage was visible in horn sections from 1 d or 5 to 6 mo old calves, but ectopic cartilage was observed in some of the ~1.5 yr old horn and scur sections. Lack of expression of cartilage markers in the horn is supporting evidence that horns form by intramembranous ossification.

Taken together, these data suggest that although horn development occurs primarily after birth, the critical stage may be the initiation of development in the embryo or fetus. We propose that the polled locus is upstream of RUNX2 and SOX9 in the osteogenic pathway, and could have its primary effect on the differentiation of mesenchymal condensations. Based on their function and associated cell lineages, *IL10RB*, *SFRS15*, *C21orf66*, *OLIG1*, *OLIG2* and *HUNK* remain candidates for the polled locus and warrant further investigation at various stages of fetal development. Finally, our characterization of horn and scurs was conducted using samples from *Bos indicus* influenced cattle, and future work will be needed verify these observations in *Bos taurus* cattle.

LITERATURE CITED

Abzhanov, A., S. J. Rodda, A. P. McMahon, and C. J. Tabin. 2007. Regulation of skeletogenic differentiation in cranial dermal bone. Development 134: 3133-3144.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Altschul, S. F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped blast and psi-blast: A new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Arvas, L., M. Livaoglu, N. Karacal, E. Sozen, and B. Kara. 2007. Giant cutaneous horn with naevus sebaceus. J. Plast. Reconstr. Aesthet. Surg. 60: 1268-1269.

Asai, M., T.G. Berryere, and S. Schmutz. 2004. The scurs locus in cattle maps to bovine chromosome 19. Anim. Genet. 35: 34-9.

Barendse W., S.M. Armitage, L.M. Kossarek, A. Shalom, B.W. Kirkpatrick, A.M. Ryan, D. Clayton, L. Li, H.L. Niebergs, N. Zhang, W.M. Grosse, J. Weiss, P. Creighton, F. McCarthy, M. Ron, A.J. Teale, R. Fries, R.A. McGraw, S.S. Moore, M. Georges, M. Soller, J.E. Womack and D.J.S Hetzel. 1994. A genetic linkage map of the bovine genome. Nat. Genet. 6:227-35.

Bi, W., J.M. Deng, Z. Zhang, R.R. Behringer, and B. de Crombrugghe. 1999. Sox9 is required for cartilage formation. Nat. Genet. 22: 85-89.

Bialek, P., B. Kern, X. Yang, M. Schrock, D. Sosic, N. Hong, H. Wu, K. Yu, D.M. Ornitz, E.N. Olson, M.J. Justice, and G. Karsenty. 2004. A Twist code determines the onset of osteoblast differentiation. Dev. Cell. 6: 423-435.

Bishop M.D., S.M. Kappes, and J.W. Keele. 1994. A genetic map for cattle. Genetics 136:619-39.

Brenneman, R.A., S.K. Davis, J.O. Sanders, B.M. Burns, T.C. Wheeler, J.W. Turner, and J.F. Taylor. 1996. The polled locus maps to BTA1 in a *Bos indicus* x *Bos taurus* cross. J. Hered. 87: 156-161.

Bricker, R.A. and R.B. Church. 1991. Mapping a polled gene in a 1:29 Robertsonian study in Charolais cattle. Abstracts of 36th annual meeting of the Genetics Society of Canada, Kingston, Ontario.

Buchanan Smith, A.D. 1927. The inheritance of horns in cattle. Some further data. J. Genet. 18: 365-374.

Cai, L. J.F. Taylor, R.A. Wing, D.S. Gallagher, S.S. Woo and S.K. Davis. 1995. Construction and characterization of a bovine artificial chromosome library. Genomics 29: 413-425.

Chen, W., C.C. Yang, C.Y. Liao, C.L. Hung, S.J. Tsai, K.F. Chen, H.M. Sheu, and C.C. Zouboulis. 2006. Expression of sex-determining genes in human sebaceous glands and their possible role in the pathogenesis of acne. J. Eur. Acad. Dermatol. Venerol. 20: 846-852.

Chen, Y. I., R.E. Moore, H.Y. Ge, M.K. Young, T.D. Lee, and S.W. Stevens. 2007. Proteomic analysis of in vivo-assembled pre-mRNA splicing complexes expands the catalog of participating factors. Nucleic Acids Res 35: 3928-3944.

Churchill, O.O. 1927. Sex and horns in cattle- A note on an exceptional mode of inheritance. J. Hered. 18: 279-280.

Davis, S.L.F., J.F. Taylor, D.R. Stillwell, C.A. Gill, and S.K. Davis. 1999. Comparative sequence analysis of the Down syndrome critical region in humans and cows identifies genes encoding rare transcripts. Page 88 in Proceedings of the genome mapping, sequencing and biology meeting, Cold Spring Harbor, NY.

Day, T.F. X. Guo, L. Garrett-Beal, and Y. Yang. 2005. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev. Cell. 8:739-750.

de Crombrugghe, B., V. Lefebvre, and K. Nakashima. 2001. Regulatory mechanisms in the pathways of cartilage and bone formation. Curr. Opin. Cell Biol. 13: 721-727.

de Jong, P.J. 2007. CHORI-240 bovine BAC library (male). Available at: http://www.chori.org/bovine240.htm Accessed July 12, 2007.

Donnelly, R.P., F. Sheikh, S.V. Kotenko, and H. Dickensheets. 2004. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10 R2) chain. J. Leukoc. Biol. 76: 314-321

Dove, F.A. 1935. The physiology of horn growth: A study of the morphogenesis, the interaction of tissues, and the evolutionary processes of a Mendelian recessive character by means of transplantation of tissues. J. Exp. Zool. 69: 347-405.

Dresner-Pollak, R., N. Gelb, D. Rachmilewitz, F. Karmeli, and M. Weinreb. 2004. Interleukin 10-deficient mice develop osteopenia, decreased bone formation, and mechanical fragility of long bones. Gastroenterology 127: 792-801.

Drögemüller, C., A. Bader, A. Wöhlke, H. Kuiper, T. Leeb, and O. Distl. 2002. A high-resolution comparative RH map of the proximal part of bovine chromosome 1. Anim. Genet. 33: 271-279.

Drögemüller, C., A. Wöhlke, S. Mömke, and O. Distl. 2005a. Fine mapping the polled locus to a 1-Mb region on bovine chromosome 1q12. Mamm. Genome 16: 613-620.

Drögemüller, C., A. Wöhlke, T. Leeb, and O. Distl. 2005b. A 4 Mb high resolution BAC contig on bovine chromosome 1q12 and comparative analysis with human chromosome 21q22. Comp. Funct. Genom. 6: 194-203.

Eames, B. F. and J. A. Helms. 2004. Conserved molecular program regulating cranial and appendicular skeletogenesis. Dev. Dyn. 231: 4-13.

Ewing, B. and P. Green. 1998. Base-calling of automated sequencer traces using *Phred*. II. Error probabilities. Genome Res. 8: 186-194.

Ewing, B., L. Hillier, M.C. Wendl, and P. Green. 1998. Base-calling of automated sequencer traces using *Phred*. I. Accuracy assessment. Genome Res. 8: 175-185.

Fambach, O. 1901. Untersuchungen und Beobachtungen über das Os cornu. Zeitschr. F. Naturwissensch., Bd. 74, S.1.

Fairbanks, D.J. and W.R. Anderson. 1999. Genetics: the continuity of life. Brooks/Cole Publishing. Pacific Grove, CA.

Feng, X. 2005. RANKing intracellular signaling in osteoclasts. IUBMB Life 57: 389-395.

Fjell C.D., I. Bosdet, J.E. Schein, S.J. Jones and M.A. Marra. 2003. Internet Contig Explorer (iCE)- a tool for visualizing clone fingerprint maps. Genome Res. 13: 1244-1249.

Fleige, S. and Pfaffl, M.W. 2006. RNA integrity and the effect on the real-time qRT-PCR performance. Mol. Aspects Med. 27: 126-139.

Flores, M.V., E.Y.N. Lam, P. Crosier, and K. Crosier. 2006. A hierarcy of Runx transcription factors modulate the onset of chondrogenesis in craniofacial endochondral bones in zebrafish. Dev. Dyn. 235: 3166-3176.

Fomenkov, A., Y. Huang, O. Topaloglu, A. Brechman, M. Osada, T. Fomenkova, E. Yuriditsky, B. Trink, D. Sidransky, and E. Ratovitski. 2003. p63α Mutations lead to aberrant splicing of keratinocyte growth factor receptor in the Hay-Wells syndrome. J. Biol. Chem. 278:23906-23914.

Gadow, H. 1902. The evolution of horns and antlers. Proc. Zool. Soc. London Pt. 1: 206-222.

Gaile, D.P. 2003. Development of statistical tools with applications in radiation hybrid and linkage mapping. Ph.D. Dissertation. Texas A&M University. College Station, Texas

García-López , S., M.C. Meikle, R.E. Villanueva, L. Montaño, F. Massó, V. Ramírez-Amador, and R. Bojalil. 2005. Mechanical deformation inhibits IL-10 and

stimulates IL-12 production by mouse calvarial osteoblasts in vitro. Arch. Oral Biol. 50: 449-452

Gardner, H.P., G.K. Belka, G.B.W. Wertheim, J.L. Hartman, S.I. Ha, P.A. Gimotty, S.T. Marquis, and L.A. Chodosh. 2000. Developmental role of the SNF1-related kinase Hunk in pregnancy-induces changes in the mammary gland. Development 127: 4493-4509.

Georges, M., R. Drinkwater, T. King, A. Mishra, S.S. Moore, D. Nielson, L.S. Sargeant, A. Sorensen, M.R. Steele, X. Zhao, J.E. Womack, and J. Hetzel. 1993. Microsatellite mapping of a gene affecting horn development in *Bos taurus*. Nat. Genet. 4: 206-210.

Gibbs, R., G. Weinstock, S. Kappes, L. Schook, L. Skow, and J. Womack. 2002. Bovine genomic sequencing initiative. www.genome.gov/10002154 Accessed February 7, 2008.

Gilbert, S.F. 1997. Developmental Biology. 7th ed. Sinauer Associates, Inc., Sunderland, MA.

Goldring, M.B., K. Tsuchimochi, and K. Ijiri. 2006. The control of chondrogenesis. J. Cell Biochem. 97: 33-44.

Goonewardene, L.A. and R.K. Hand. 1991. Studies on dehorning steers in Alberta feedlots. Can. J. Anim. Sci. 71: 1249-1252.

Goonewardene, L.A., M.A. Price, M.F. Liu, R.T. Berg, and C.M. Erichsen. 1999. A study of growth and carcass traits in dehorned and polled composite bulls. Can. J. Anim. Sci. 79: 383-385.

Goosens, K., M. Van Poucke, A. Van Soom, J. Vandesomplete, A. Van Zeveren, and L.J. Peelman. 2005. Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. BMC Dev. Biol. 5:27.

Gordon, D., C. Abajian, and P. Green. 1998. Consed: A graphical tool for sequence finishing. Genome Res. 8: 195-202.

Graphodatskaya, D., J. Hannes, M. Asai-Coakwell, F. Janet, and G. Stranzinger. 2006. Expression and function of agouti signaling protein in cattle. Anim. Sci. J. 77: 33-41.

Harlizius B., J. Hetzel, and W. Barendse. 1995. Comparative mapping of the proximal part of bovine chromosome 1. Mamm. Genome. 6: 481-483.

Harlizius, B., I. Tammen, K. Eichler, A. Eggen, and D.J.S. Hetzel. 1997. New markers on bovine chromosome 1 are closely linked to the polled gene in Simmental and Pinzgauer cattle. Mamm. Genome 8: 255-257.

Hu, Y.H., H.J. Warnatz, D. Vanhecke, F. Wagner, A. Fiebitz, S. Thamm, P. Kahlem, H. Lehrach, M.L. Yaspo, and M. Janitz. 2006. Cell array-based intracellular localization screening reveals novel functional features of human chromosome 21 proteins. BMC Genomics 7: 155.

Huang, Y-P., Y. Kim, Z. Li, T. Fomenkov, A. Fomenkov, and E.A. Ratovitski. 2005. AEC-associated p63 mutations lead to alternative splicing/protein stabilization of p63 and modulation of notch signaling. Cell Cycle 4: 1440-1447.

Itoh, S., N. Udagawa, N. Takahashi, F. Yoshitake, H. Narita, S. Ebisu, and K. Ishihara. 2006. A critical role for interleukin-6 family mediated Stat3 activation is osteoblast differentiation and bone formation. Bone. 39: 505-512.

Jakovcevski, I., and N. Zecevic. 2005. Olig transcription factors are expressed in oligodendrocyte and neuronal cells in human fetal CNS. J. Neurosci. 25: 10064-10073.

Karsenty, G. 2001a. Genetic control of skeletal development. Novartis Found. Symp. 232: 6-17; discussion 17-22.

Karsenty, G. 2001b. Minireview: Transcriptional control of osteoblast differentiation. Endocrinology. 142: 2731-2733.

Kartsogiannis, V., J. Moseley, B. McKelvie, S.T. Chou, D.K. Hards, K.W. Ng, T.J. Martin, and H. Zhou. 1997. Temporal expression of Pthrp during endochondral bone formation in mouse and intramembranous bone formation in an in vivo rabbit model. Bone 21: 385-392.

Kim, S., T. Koga, M. Isobe, B.E. Kern, T. Yokochi, Y.E. Chin, G. Karsenty, T. Taniguchi, and H. Takayanagi. 2003. Stat1 functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation. Genes Dev. 17: 1979-1991.

Korobko, E. V., S. L. Kiselev, and I. V. Korobko. 2004. Subcellular localization of Mak-V/Hunk protein kinase expressed in Cos-1 cells. Cell Biol. Int. 28: 49-56.

Kronenberg, H.M. and U. Chung. 2001. The parathyroid hormone-related protein and Indian hedgehog feedback loop in the growth plate. Novartis Found. Symp. 232: 144-152; discussion 152-157.

Kronenberg, H. M. 2003. Developmental regulation of the growth plate. Nature 423: 332-336.

Lampasso, J. D., N. Marzec, J. Margarone, 3rd, and R. Dziak. 2002. Role of protein kinase C alpha in primary human osteoblast proliferation. J. Bone Miner. Res. 17: 1968-1976.

Lange, H., G. Brem, J. Utz, A. Gottschalk, B. Karnbaum, and H. Kräusslich. 1990. Untersuchungen über Hornlosigkeit beim Desutschen Fleckvieh. Bayerisches Landwirtschaftliches Jahrbuch Heft. 67: 15-68.

Lefebvre, V., R. R. Behringer, and B. de Crombrugghe. 2001. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage 9 Suppl A: S69-75.

Li, X., H. Wang, E. Touma, E. Rousseau, R.J. Quigg, and J.T. Ryaby. 2007. Genetic network and pathway analysis of differentially expressed proteins during critical cellular events in fracture repair. J. Cell Biochem. 100: 527-543.

Lincoln, S.E., M.J. Daly, and E.S. Lander. 1991. Primer 0.5 Computer Software. MIT Center for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, MA.

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408.

Long, C.R., and K.E. Gregory. 1978. Inheritance of the horned, scurred, and polled condition in cattle. J. Hered. 69: 395-400.

Luo, M., C. Thomas, F.M. You, J. Hsiao, S. Ouyang, C.R. Buell, M. Malandro, P.E. McGuire, O.D. Anderson, and J. Dvorak. 2003. High-throughput fingerprinting of bacterial artifical chromosomes using the SNaPshot labeling kit and sizing of restriction fragments by capillary electrophoresis. Genomics 82: 378-389.

Mackie, E. J., Y. A. Ahmed, L. Tatarczuch, K. S. Chen, and M. Mirams. 2007. Endochondral ossification: How cartilage is converted into bone in the developing skeleton. Int. J. Biochem. Cell Biol. 40: 46-62.

Marques F.L.S., R.A. Brenneman, S.M. Schmutz, J.F. Taylor, and S.K. Davis. 1997. A highly polymorphic bovine dinucleotide repeat SOD1MICRO2. Anim. Genet. 28:58-71.

McKenna, D.R., D.L. Roeber, P.K. Bates, T.B. Schmidt, D.S. Hale, D.B. Griffin, J.W. Savell, J.C. Brooks, J.B. Morgan, T.H. Montgomery, K.E. Belk, and G.C. Smith. 2002. National beef quality audit-2000: Survey of target cattle and carcass characteristics related to quality, quantity, and value of fed steers and heifers. J. Anim. Sci. 80: 1212-1222.

Mills, A.A., B. Zheng, X. Wang, H. Vogell, D.R. Roop, and A. Bradley. 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398: 708-13.

Nakashima, K., and B. de Crombrugghe. 2003. Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet. 19: 458-466.

Neter, J. and W. Wasserman. 1974. Applied Linear Statistical Models. Richard D. Irwin, Inc., Homewood, IL.

Ng, L. J., S. Wheatley, G.E. Muscat, J. Conway-Campbell, J. Bowles, E. Wright, D.M. Bell, P.P. Tam, K.S. Cheah, and P. Koopman. 1997. Sox9 binds DNA, activates transcription, and coexpresses with type ii collagen during chondrogenesis in the mouse. Dev. Biol. 183: 108-121.

Nie, X., K. Luukko, I.H. Kvinnsland, and P. Kettunen. Developmentally regulated expression of Shh and Ihh in the developing mouse cranial base: Comparison with Sox9 expression. Anat. Rec. A. 286: 891-898.

Oh, C. D., S.J. Kim, J.W. Ju, W.K. Song, J.H. Kim, Y.J. Yoo, J.S. Chun. 2001. Immunosuppressant rapamycin inhibits protein kinase C alpha and p38 mitogen-activated protein kinase leading to the inhibition of chondrogenesis. Eur. J. Pharmacol. 427: 175-185.

Ornitz, D. M., and P. J. Marie. 2002. Fgf signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes Dev. 16: 1446-1465.

Ottolenghi, C., E. Pelosi, J. Tran, M. Colombino, E. Douglass, T. Nedorezov, A. Cao, A. Forabosco, and D. Schlessinger. 2007. Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. Hum Mol Genet 16: 2795-2804.

Pasquini, C. 1982. An Atlas of Bovine Anatomy. Sudz Publishing, Pilot Point, TX.

Pizette, S., and L. Niswander. 2001. Early steps in limb patterning and chondrogenesis. Novartis Found. Symp. 232: 23-36; discussion 36-46.

Pailhoux, E., B. Vigier, S. Chaffaux, N. Servel, S. Taourit, J.P. Furet, M. Fellous, F. Grosclaude, E.P. Cribiu, C. Cotinot, and D. Vaiman. 2001. A 11.7-kb deletion triggers intersexuality and polledness in goats. Nat. Genet. 29: 453-458.

Radeff, J. M., A. T. Singh, and P. H. Stern. 2004. Role of Protein kinase A, Phospholipase C and Phospholipase D in parathyroid hormone receptor regulation of Protein kinase C alpha and interleukin-6 in UMR-106 osteoblastic cells. Cell Signal 16: 105-114.

Rappsilber, J., U. Ryder, A. I. Lamond, and M. Mann. 2002. Large-scale proteomic analysis of the human spliceosome. Genome Res. 12: 1231-1245.

Rexroad, C. E., J. S. Schlapfer, Y. Yang, B. Harlizius, and J. E. Womack. 1999. A radiation hybrid map of bovine chromosome one. Anim. Genet. 30: 325-332.

Reymond, A., M. Friedli, C.N. Henrichsen, F. Chapot, S. Deutsch, C. Ucla, C. Rossier, R. Lyle, M. Guipponi, and S.E. Antonarakis. 2001. From PREDs and open reading frames to cDNA isolation: revisiting the human chromosome 21 transcription map. Genomics 78: 46-54.

Roeber, D.L., P.D. Mies, C.D. Smith, K.E. Belk, T.G. Field, J.D. Tatum, J.A. Scanga, and G.C. Smith. 2000. National market-cow and bull beef quality audit-1999: A survey of producer-related defects in market cows and bulls. J. Anim. Sci. 79: 658-665.

Roers, A., L. Siewe, E. Strittmatter, M. Deckert, D. Schlüter, W. Stenzel, A.D. Gruber, T. Krieg, K. Rajewsky, and W. Müller. 2004. T cell-specific inactivation of the

interleukin 10 gene in mice results in enhanced responses but normal innate responses to lipopolysaccharide or skin irritation. J. Exp. Med. 200: 1289-1297.

Schmutz, S.M., F.L.S. Marquess, T.G. Berryere, and J.S. Moker. 1995. DNA marker-assisted selection of the polled condition in Charolais cattle. Mamm. Genome. 6: 710-713.

Slavov, D., M. Hattori, Y. Sakaki, A. Rosenthal, N. Shimuzi, S. Minoshima, J. Kudoh, M.L. Yaspo, J. Ramser, R. Reinhardt, C. Reimer, K. Clancy, A. Ryndictch, and K. Gardiner. 2000. Criteria for gene identification and features of genome organization: Analysis of 6.5 Mb of DNA sequence from human chromosome 21. Gene 247: 215-232.

Smith, N., Y. Dong, J.B. Lian, J. Pratap, P.D. Kingsley, A.J. van Wijnen, J.L. Stein, E.M. Schwarz, R.J. O'Keefe, G.S. Stein, and M.H. Drissi. 2005. Overlapping expression of Runx1(Cbfa2) and Runx2(Cbfa1) transcription factors supports cooperative induction of skeletal development. J. Cell Physiol. 203: 133-143.

Smith, G. C., J. W. Savell, J. B. Morgan, and T. E. Lawrence. 2006. Report of the June-September, 2005 National Beef Quality Audit: A new benchmark for the U. S. beef industry. Proceedings of the 38th Annual Meeting of the Beef Improvement Federation. http://www.bifconference.com/bif2006/pdfs/Morgan.pdf Accessed Feb. 14, 2008.

Snelling, W. M., R. Chiu, J. E. Schein, M. Hobbs, C. A. Abbey, D. L. Adelson, G. L. Bennett, I. E. Bosdet, M. Boussaha, R. Brauning, A. R. Caetano, M. M. Costa, A. M. Crawford, B. P. Dalrymple, A. Eggen, A. Everts-van der Wind, S. Floriot, M.

Gautier, C. A. Gill, R. D. Green, R. Holt, S. J. M. Jones, S. M. Kappes, J. W. Keele, P. J. de Jong, D. M. Larkin, H. A. Lewin, J. C. McEwan, S. McKay, S. McWilliam, M. A. Marra, C. A. Mathewson, L. K. Matukumalli, S. S. Moore, B. Murdoch, F. Nicholas, K. Osoegawa, A. Roy, H. Salih, L. Schibler, R. Schnabel, L. Silveri, L. C. Skow, T. P. L. Smith, T. S. Sonstegard, J. Taylor, R. Tellam, C. P. Van Tassell, J. L. Williams, J. E. Womack, N. H. Wye, G. Yang, S. Zhao. 2007. A physical map of the bovine genome. Genome Biol. 8: R165.

Song, Y. 1998. Genetic and physical fine mapping of the POLL locus to BTA1 in a *Bos indicus* x *Bos taurus* cross. M.S. Thesis. Texas A&M University. College Station, Texas.

Stillwell, D. 1998. Genetic and physical mapping of the POLL locus to bovine chromosome 1 in a *Bos indicus* x *Bos taurus* cross. M.S. Thesis. Texas A&M University. College Station, Texas

Takahashi, N., G. R. Moody, and G. D. Goodman. 1986. Recombinant human interferon-gamma inhibits formation of human osteoclast-like cells. J. Immunol. 137: 3544-3549.

Takayanagi, H. S. Kim, K. Matsuo, H. Suzuki, T. Sunuki, K. Sato, T. Yokochi, H. Oda, K. Nakamura, N. Ida, and E.F. Wagner. 2002. RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. Nature 416: 744-749.

Takeda, H. and Y. Sugimoto. 2003. Construction of YAC/BAC contig map for the BTA 6q21 region containing a locus for bovine chondrodysplastic dwarfism. Anim. Biotechnol. 14: 51-59.

Ueta, C., M. Iwamoto, N. Kanatani, C. Yoshida, Y. Liu, M. Enomoto-Iwamoto, T. Ohmori, H. Enomoto, K. Nakata, K. Takada, K. Kurisu, T. Komori. 2004. Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. J. Cell Biol. 153: 87-99.

Van Vlasselaer, P., B. Borremans, U. Van Gorp, J.R. Dasch, and R. De Waal-Malefyt. 1994. Interleukin 10 inhibits transforming growth factor-β (TGF-β) synthesis required for osteogenic commitment of mouse bone marrow cells. J. Cell Biol. 124: 569-572.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.

Vidal, V.P., M.C. Chaboissier, S. Lützkendorf, G. Cotsarelis, P. Mill, C.C. Hui, N. Ortonne, J.P. Ortonne, and A. Schedl. 2005. Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment. Curr. Biol. 15: 1340-51.

Visel A., C. Thaller, and G. Eichele. 2004. Genepaint.org: An atlas of gene expression patterns in the mouse embryo. Nucleic Acids Res. 32:D552-6.

Wang, D. G., J.-B. Fan, C.-J. Siao, A. Berno, P. Young, R. Sapolsky, G. Ghandour, N. Perkins, E. Winchester, J. Spencer, L. Kruglyak, L. Stein, L. Hsie, T. Topaloglou, E. Hubbell, E. Robinson, M. Mittmann, M. S. Morris, N. Shen, D. Kilburn, J. Rioux, C. Nusbaum, S. Rozen, T. J. Hudson, R. Lipshutz, M. Chee, and E. S. Lander. 1998. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science. 280:1077-1082.

White, W.T., and H.L. Ibsen. 1936. Horn inheritance in Galloway-Holstein cattle crosses. J. Genet. 11: 59-67.

Williams, H.D. and T. Williams. 1952. The inheritance of horns and their modifications in polled Hereford cattle. J. Hered. 43: 267-272.

Winter, A., A. Alzinger, and R. Fries. 2004. Assessment of the gene content of the chromosomal regions flanking bovine *DGAT1*. Genomics 83: 172-180.

Wunderlich, K.R. C.A. Abbey, D.R. Clayton, Y. Song, J.E. Schein, M. Georges, W. Coppieters, D.L. Adelson, J.F. Taylor, S.L. Davis, and C.A. Gill. 2006. A 2.5-Mb contig constructed from Angus, Longhorn and horned Hereford DNA spanning the polled interval on bovine chromosome 1. Anim. Genet. 37: 592-594.

Xiao, L., T. Naganawa, E. Obugunde, G. Gronowicz, D.M. Ornitz, J.D. Coffin, and M.M. Hurley. 2004. Stat1 controls postnatal bone formation by regulating fibroblast growth factor signaling in osteoblasts. J. Biol. Chem. 279: 27743-27752.

Xu, L. X., T. Kukita, A. Kukita, T. Otsuka, Y. Niho, T. Iijima. 1995. Interleukin-10 selectively inhibits osteoclastogenesis by inhibiting differentiation of osteoclast progenitors into preosteoclast-like cells in rat bone marrow culture system. J. Cell Physiol. 165: 624-629.

Yousfi, M., F. Lasmoles, A. Lomri, P. Delannoy, and P. J. Marie. 2001. Increased bone formation and decreased osteocalcin expression induced by reduced Twist dosage in Saethre-Chotzen syndrome. J. Clin. Invest. 107: 1153-1161.

Yuryev, A., M. Patturajan, Y. Litingtung, R. V. Joshi, C. Gentile, M. Gebara, J. L. Corden. 1996. The C-terminal domain of the largest subunit of RNA polymerase II interacts with a novel set of serine/arginine-rich proteins. Proc. Natl. Acad. Sci. USA 93: 6975-6980.

Zhao, Q., P. R. Brauer, L. Xiao, M. H. McGuire, and J. A. Yee. 2002. Expression of parathyroid hormone-related peptide (Pthrp) and its receptor (Pth1r) during the histogenesis of cartilage and bone in the chicken mandibular process. J. Anat. 201: 137-151.

APPENDIX A

Table A.1. Primers used to screen the TAMBT and CHORI-240 BAC libraries

Marker	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)	Tm (ºC)
AGLA17[1]		AATAGACCTAGTCTGAACCGAG	GTTTAAAATTTTCTAGTAACCATGT	214	53
BM6438[2]		TTGAGCACAGACACAGACTGG	ACTGAATGCCTCCTTTGTGC	258	57
C21ORF45_STS1	DQ886329	TTGTACTGCACAGGATGTTCG	GGCAGAACAAGTCCCTCTTG	88	57
C21ORF45_STS2	DQ886298	CGGATTGTTGTGAAGGATGT	ACAGCGTAGCAAGATGCAGT	323	58
C21ORF59_STS	DQ886296	CCATCATTAGCAGCGAGGAG	GGCAAACTTCATCTGGGTCT	476	58
C21ORF62_STS	DQ886332	AACAGAGTTTGCTCATCCAC	GGCACAGCTACGATATTAGTAA	263	55
C21ORF63_STS1	DQ886334	CTGTTCGTGTCCAGTGTCTG	TCTTCATTCTGGCTGTCCTC	155	57
C21ORF63_STS2	DQ886333	TGACCCATCAGTTGCTAATC	CAGAAAGGCATGTATGGATG	437	57
GCFC_STS1	DQ886295	GATGTCGATGTCGCACTGTT	CTGCATTCACCACTGAAGGA	825	55
GCFC_STS2	DQ886296	AGRATGAGAAGAACTTTAGATGA	CAGAATTTYTATTTTCTAAGAC	448	53
HUNK_STS1	DQ886337	TTGATGGAACATGCCAGAAC	TCTGAATCTGCGTGATGAGC	373	57
HUNK_STS2	DQ886345	GATGATCCGCCACCCTAA	AGGTGCTCCACTGCAGAGAT	183	53
IFNAR[3]		AGAAGTTTTCTGCGTCCTTTGCC	TGATGGTGGTATTCAGGTTCTTC	290	55
IFNAR1_STS	DQ886350	GTGGTCATTTATGTTGTGAGC	GAAGTGGAAAGGAGTAGATTCC	1358	55
IFNAR2_STS	DQ886339	ATTCACRTCAMCAGGAAGAAGAA	CACACAGAGTTCARATTCACRTT	398	55
IL10RB_STS1	DQ886352	TGATGAGAGTTCAGAGTGGA	CATGTAAAGAATTAGCAAGTGC	1600	56
IL10RB_STS2	DQ886307	CACCYCCYGARAAKGTCAGAA	CTTWSRTACTGAGCTGTGAAAGTCA	109	57
LOC526226_STS	DQ886349	TACATGGGTCGCGCATCT	TGATTGAGGTGTCCTCCTGG	98	57
MRAP_STS	DQ886299	CATCTGGAGGCAACCCAG	CTCCCATCGAGAGGTTCTGT	234	58
MS204758	DQ886354	GAAAGCAGAGATGTCAAAGATGC	CAGTTAGGCACATTTAAGGGTCA	147	52
OLIG1_STS	DQ886344	GGCGCAAGATCAACAGC	CCAGCAGCAGGATGTAGTTG	174	64
OLIG2_STS	DQ886274	CGCAAGCGCATGCACGACCTYAA	CRCAGGCCGACGGGTGGAARCC	217	62
SFRS15_STS	DQ886300	AGTTCGTGTATTGAACCTTTG	GGTACAGTTGGTACAGAGTTTG	525	58
SLC5A3_STS	DQ886346	CGCTCTATGACCTGGGTAG	TCTGTGTAGATTACTGCGACAAG	425	55
SOD1[4]		GTTTGGCCTGTGGTGTAATTGGAA	GGCCAAAATACAGAGATGAATGAA	150	55
SOD1M1[5]	DQ886308	CCAAAAAACCAAAACATAAA	GGCTTACATAGTCCAATCAA	138	55
SOD1M2[5]	DQ886347	AGGGCTACAGTCCACGGGTTG	AGCGATTCACAGTCACCTCACCTA	143	64
SYNJ1_STS	DQ886348	AGTTGCAATACGAATGCTGT	TGCTGTCTGAGAAGCTCTTTC	471	64
TAMU109	DQ886294	CCCCTTCGCTTCTATGAGTCTC	GTAGTCCCGCCGATGGTT	195	57
TAMU199	DQ886313	GCACCCCTATGTTCATTGTA	GGTCCATCCACATTGTCAT	183	57
TAMU200	DQ886342	AATGAAATAACTTAGCACC	GCCTGTTGCAGCAT	175	55

Table A.1. Continued

Marker	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)	Tm (ºC)
TAMU201	DQ886340	CGCAAAACCCAGAAATG	CATCACAAATAAGCCAGCAA	269	55
TAMU202	DQ886338	CCTCCTGTTTACCTCTGAC	AAATAAGCCACGAAAACACA	161	56
TAMU203	DQ886309	GCCACCTGGTGTGAA	GACAAGAGGTTCCCAGTATT	268	57
TAMU204	DQ886341	AGTCATGGGGTCAAGCAT	CCCGTCTTCCATCATTTTAG	291	53
TAMU205	DQ886275	CCTTGCGGGGCAGCCAA	GCCGAGGCACTGCTGTCCAA	145	53
TAMU207	DQ886276	AGTTGGACATGACTGAGTGACCA	TCCCAAAGAGTCGGGCATAAC	185	62
TAMU208	DQ886277	CTTGACAGAGCCTCTAATGTGGAA	ACAGCATAATAACAGAGCTGGGAC	206	55
TAMU210	DQ886279	CACTCGCAGCATTGCGCCACCT	CCAGGCCAGCTTTTGGTCTGTTCG	247	57
TAMU211	DQ886280	GAGGGATGGACTGGGAGTTTGATT	AGTTTCTGTGGTGCTGCAAAGTGA	237	55
TAMU212	DQ886281	CTGGTGTTCCTCTGGCCACGGG	TCCCAGCCTCTGGGGTGACTGCC	212	62
TAMU215	DQ886286	CCCTGATGTAGGCTCAATAAG	TCACATCCTGCATTTGGTAG	177	55
TAMU216	DQ886284	ACACATCTATTGGGAAAATGC	AGCCTACATCAGGGAAGGT	124	55
TAMU218	DQ886289	CAACTTACGTCCGCCATAACAA	GAAAAATGAAGCCTGCCACAC	123	55
TAMU219	DQ886290	ACTGAGTCATCGATGTCTCAT	CTCAGAGGAGGATATTGCAAG	237	57
TAMU220	DQ886292	TCCAGTCCTCTGCCCATTG	CTTTGCGAAACCTCCCTTACAG	162	60
TAMU221	DQ886293	CCCTGCATTCGGCTGACT	TGGCAGAAAATTCACACCTTCC	145	55
TAMU222	DQ886331	GGCAAAAAGTTGGACA	GAGGTAGACCTAGAAGAGGC	169	62
TAMU223	DQ886330	AACTGGAAAGAAGAGGCATC	CACCTGGGGAATCCTGAT	162	57
TAMU224	DQ886304	ATAAGTTCTGGATTTGTGGTTC	CCTCCAGTGAATGTCTTTGT	162	52
TAMU225	DQ886336	GGAGACATTCCATAAACAGA	TCTACTGGTTCCTTGACATTC	149	53
TAMU226	DQ886305	CACTACAATGTTCTTGCCTG	TCACAAAGTTTTAGTGTTAGCA	165	52
TAMU227	DQ886306	AAGAGTACTGGAGTGAGTTGC	CAGTCCTTCCTAGTTAGAGTCA	196	52
TXAG92	DQ886314	CAATAAGCGAGGTTCCTGAA	AGGGTCTGTTGAGCTGACAG	187	58
TXAG93	DQ886324	CTGGGCTCCTCCTCTTATTC	TCGTGTCTCAGTGATTTCGA	131	58
TXAG94	DQ886319	TGTGCCTATTTCTGCAACAA	CCTACAGCATTCTGCTTGGT	315	58
TXAG95	DQ886315	CTCCCAGCTTTGAAGAATCA	AGTGGGCAGAGAAGACAGTG	175	58
TXAG96	DQ886322	CTGCAGCCTTGAAAGACAAG	CACGCATTTGGCATTTTG	159	58
TXAG97	DQ886323	GGCAAAGTTTTGTCTTCAGC	GCCAAGTTCTATGTCATGGG	194	58
TXAG98	DQ886320	AACAGAAACAGAAACCTGCG	TTGATGCTTGGGTACCAACT	163	58
TXAG99	DQ886321	ATCATTAGAACCCTCCTGGG	TCCATCTTTTGGAAATCTGC	163	58

Table A.1. Continued

Marker	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)	Tm (ºC)
TXAG100	DQ886301	TAACAGGAATTGAACCTGGC	TAGCGATGACCAAAACACAC	204	57
TXAG101	DQ886318	GGTAGGTACTTTGGGCATCA	TGGTGGGCAGAAGAGC	384	62
TXAG102	DQ886335	AGAACACAGCTGACTTTGGG	TAGGACTGAGGACTTGCACC	233	55
TXAG103	DQ886327	CAGTACACATTTGAGGAAGTGGGTT	GCTGGTATCTGCTTCCTGTAGTTCA	388	57
TXAG104	DQ886311	ACACTCTGCACCATGAATCTG	TGTTCTGGTTGGACGGTTGT	157	55
TXAG105	DQ886326	GTCTCTGCCCATTTATAATTTCTGC	TGTCGGGATGAGCTATTTGGT	356	55
TXAG107	DQ886312	TTGCAGTTTAATTAGGTACTT	ACTGGCTACTTCCTAACAG	208	57
TXAG108	DQ886302	CTAAGTCTGCTCAGGCTGGTA	TCAGAACSTGGATTGTGGG	110	55
TXAG110	DQ886328	CAATTTAGCGCTGTTTCTAT	AGGCCAGGCTGTATTC	152	55
TXAG111	DQ886317	GTTTTGCACATACATTCA	GATCCAGCAATCTAACACTA	394	57
TXAG112	DQ886343	CTTCCAGTCCCTTCTACACC	TAAGCCAGCTCTCCTGACTC	664	60
TXAG113	DQ886316	CCCCCACTTCTACCCATAA	AGGCCATAGGAGAGATGAGT	317	57
TXAG114	DQ886325	TTACATGCTTCCTGCTGTGA	TTGCACTTCTGTCAGCTTTG	128	58
TXAG118	DQ886310	CTGAACAGACCTGGACAGCT	TACCTCAAGTTGTCATGGCA	138	52
TXAG119	DQ886303	AAGGGCTCTACCAGGAAAGA	TCTTTCACAGAGTTCATTGACG	122	55
TXAG209	DQ886278	TCCCAAACTCCCTAACTATGAATA	GGCATTGACCTCTTCTCCC	246	55
TXAG213	DQ886282	GATAATTTATGGAAAACCCTTA	ATCTCTCAGCTGCTTTATTGTT	609	55
TXAG214	DQ886283	ATAATACTCGCTTCTCTAAAG	CAACTGACCTCAAACCT	320	55
TXAG217	DQ886288	AAATCAGGTTGGAGCCGTCTC	GTGTTCCCAAAGCGAATGC	324	55

[1]Georges et al., 1993.

[2]Bishop et al., 1994.

[3]Harlizius et al., 1995.

[4]Barendse et al., 1994.

[5]Marques et al., 1997.

Table A.2. Breed Panel Samples used to sequence *C21orf66*

Sample	Name/ Data on Semen Straw
AK1	Challenger 8 Drake-Ankole AS159WA14 4-28-03
AK2	ASI 59WA Duke Watusi 58 APV6242 9-2-82
AK3	ASI 59WA08 Ankole #2 Drake Watusi
AK4	ASI 59 WA04 Ankole #4 Watusi Drake 3-17-81
AK5	WSU AKEBONO 28KB0004 AWAPB596 PHN W101 5-7-92
AK6	ASI 59 WA15 COLASSAL 9 DRAKE-ANKOLE 4-28-83
AK7	ASI 59WA25 PERFECTION ANSLEY ANKOLE 6-1-83
AK8	52 WA001 WATUSI 1 NOT REG. DICKINSON 4-29-04
AN1	SS ANGUS RITO 616 OF 48206807 12530601 032800 TAN194
AN2	04-08-99 12778058 MARCY'S POWERSTROKE 257 90AN4969
AN3	11897630 4-27-99 HSAF ANGUS 158-AN-130
AN4	CSU AGGIE EMULATION 11889421 070600
AN5	54AN1469 OGL BATTLE CRY 427 128 12215320 11-18-98 WHITESTONE-KREBS
AN6	115448243 VDAR BANDO 701 536 14AN0174 06/14/99
AN7	10-05-98 12240991 HIGH VALLEY 4C6 AMBUSH 90AN4309
AN8	ABS N BAR EMULATION EXT 10776479 29AN1413 98208
BR1	MAPLE BROOK RANGER 11587 45R MBR RE 82-0484 141BD0001
BR2	MR 3X MAXMILLION 100/6 459237 154BR375
BR3	SRS MR RAIDER 2BR1146 554 754942 0119901
BR4	141 BR 0089 EL DORADO 277 506341 277 4 24 90
BR5	3B MR SUVA 239/8 633216 154BR329 91152
BR6	JJ DIDOR CRATA 500 ABBA 378630 91087
BR7	POLO 628 895IH ABBA# 75146
BR8	WHS MADHYO ESTO 542/8 ABBA 271991 74881
SM1	SSSIMMENTAL DS BLACK ZINGER 1418 1629874 CSS 082294 7SM24
SM2	css 11-17-97 85s1342 srsf607 FORTUNE 500 ISM0041
SM3	12-10-98 54SM697 NICHOLS BLK DESTINY D12 1757710 CSS NICHOLS
SM4	21SM0405 1189489 JY ROLLED BOLD LEADER 91018
SM5	DS SIMMENTAL SSM282 POLLFLECK 809 1122699 CSS 10017
SM6	ABS CSS BLACK IRISH KANSAS 14225514 29SM0298 96338
SM7	ABS CSS ER BIGSKY 545B 1600900 29SM0318
SM8	11-6-90 53SM090 11-6-90 MILKMAN 1324203
HH1	WE LI DOMINO T565 AHA 18677617-87987
HH2	BR LI DOMINO 1067 A9249938 157HH624
HH3	J-F RIMFIRE 141HH048 19085132 9040 11-21-90
HH4	BB DOMINO 1087 28HH0247 B1087 2043936 10-20-83
HH5	MF 435 ADVANCER 7989 AHA 18831767-24788
HH6	STAR MARK 300C 5311 18403236
HH7	CLI DOMINO 667 56HH024 AHA 18755019
HH8	RCR L1 ENCORE 7018 AHA 18856439-22988
PH1	BEARTOOTH RANCH 54HP42 BT BUTLER 152M X21830428
PH2	SUNBURST ROUNDUP 302R 18HP138 X22741584 08274
PH3	54HP350 GK FUTURE VISION X23288472 G. RABOIN & K. STORK 12-12-90
PH4	SS P. HEREFORD JMS VICTER 2105 858 X23272893
PH5	54HP44 BT CL DOMINO 445M X21786855 2-22-83
PH6	16HP N297 X23757313 SL DOMINOS ACCLAIM 8D 12-16-97
PH7	54HP855 BARJZ PUCKSTER 892H P41056015 5-21-99 PALUMBA
PH8	T3 TRIUMPH 13 W AHA 23212139-30989

Table A.3. Angleton project grandparents used to sequence *C21orf66*

Sample	Name
AGP1	SCOTCHCAP
AGP2	W11
AGP3	T5
AGP4	P59
AGP5	G211
AGP6	868013
AGP7	SHOSHONE
AGP8	PINETAR
AGP9	POWERDRIVE
AGP10	PINEDRIVE
AGP11	SHYHIGH
AGP12	WRANGLER
AGP13	MR. ANGUS
AGP14	CALIBRATED P14
AGP15	W6
AGP16	2546
AGP17	868054
AGP18	2627
AGP19	2520
AGP20	P44
AGP21	P48
AGP22	P12
AGP23	2749
BGP1	249/3
BGP2	9/118
BGP3	ROCKY
BGP4	EJL309
BGP5	G703
BGP6	LA500
BGP7	V8777/2
BGP8	34/3
BGP9	4803
BGP10	P3385
BGP11	P3725
BGP12	2_7
BGP13	296/1
BGP14	164/3
BGP15	9_3
BGP16	P363
BGP17	1_4
BGP18	539/1
BGP19	6102
NGP1	6869
NGP2	6996
NGP3	6863

Table A.4. Primers used for PCR and sequencing of *C21orf66*. Lone primers and primers without PCR conditions were only used as sequencing primers

Primer	Forward	Reverse	Temperature	$MgCl_2$	Length
GCFC_BP1	AGTGATTGCTGAAAATATGTGG	TCAATTGGTACACATCCCAG	55	2	437
GCFC_BP2	CAGCCAGTTTCTTTACCATC	CATTCCTGTCTTCAAATGGT	55	2	487
GCFC_BP3	CTGTTTTCATCCTTCTCACC	ACTTACTTTTTGGGATACAAGG	55	1.5	624
GCFC_BP4	GATGTCGATGTCGCACTGTT	CTGCATTCACCACTGAAGGA	55	1.5	825
GCFC_BP6	CCTGTGATCCCACTAAAGAC	TGTGAATTTTCTTGTTTCAAA	58	1.5	990
GCFC_intBP6	AGATTTTCCCACAACCAGTCTCTC				
GCFC_BP7	GAGGATCACAAAAAGTTCAAC	CAAAGATTGGAGAGAACTCC	60	1.5	989
GCFC_intBP7		GCCTAACCTTATTAGTACGTCATATTTC			
GCFC_BP8[2]	AGTTCCGTTCCAATAGTCCT	CAGAATGACTGATCTTAGGTCC	55	2.5	986
GCFC_BP9	TCTCTTAACTACCGCCTCCT	CCAAAATCAAAAGGTGCTAG	55	1.5	967
GCFC_BP10	GGTCTTGAAATAACTGTGTCC	TCGAAGAATTAAAATGACTGTG	54.5	1.5	964
GCFC_BP11	AAGGAGTACTGTGCAAATTCC	CAGCAAACATTTTAAATTCACC	56.4	1.5	1014
GCFC_BP12	GCTTTGATCCAGTAAGAGTGG	CCTTCCTTTTCCTGACCTTC	60	1.5	992
GCFC_BP13	AGGGAAGTTTCAAGATGATG	CCTCTAAAGAAGAATCCTGG	58	1.5	1005
GCFC_BP14	TGAAGCAGAGTGACCTAAATC	CATCAAACTCTAAATCCATCTG	51.6	1.5	979
GCFC_BP15	ATAGAAAGCTGCCTCAAATG	TGAGAATGAACTTGAGAACTTT	61.3	1.5	950
GCFC_BP16	TAAGTGGTGTTTCTTCCGAC	TTTCACCTGCAAATAGTTTTC	50	1.5	1028
GCFC_BP17	TTCTGGGAACAAACACTTAGG	GTACTGAGAGGCAATGTAAAGC	57.6	1.5	1004
GCFC_BP18[1]	CAATCCAGATGATACAGAAGC	CACACAGCAACTGCAATATC	51.6	1.5	1407
GCFC_BP19	TTAAGTAGGGAGGTGACTTGC	CACCAGGACCTAAGTGTTTG	52	1.5	357
GCFC_BP20	GGTCCATGGTATTTCATAAGG	AACCGTCTATTGATAATTCCTG	56	1.5	1011
GCFC_BP21	AATTTTCTTCAATGGTATGGC	TTTGATGGTGAGGGAGTAAG	59.8	1.5	664
GCFC_BP22	GACCTGTACCATGTCAGAGTAA	GTATAGCATAAACACCAAATCG	60	1.5	803
GCFC_BP23	CTCTCTCCACCCTGTGCTG	TACTTGGGGTTTCAGTGGCT	67	1.5	1016
GCFC_BP24	ATCTTGGTCTTTCGAGGTTC	CACTTGCAACAGACATAGCA	58	1.5	1002
GCFC_BP25[1]	TCTGGCTACTCATCTAAACAGG	ATTTAAGCTGGTCAGGGAGT	57.6	1.5	980
GCFC_BP26	ATAGACCCACAACCTAACTGC	TGAAAGTATAAGAGCCTGATGG	59	1.5	996

Table A.4. Continued

Primer	Forward	Reverse	Temperature	$MgCl_2$	Length
GCFC_BP27	TTCGAAACCTATTGGACTGA	TTGTGAGAGATGACACGGAT	56	1.5	1012
GCFC_BP28	TTCACGTTGAACATACCAGC	AAGTAACAGCCCCTGAACTG	57	1.5	808
GCFC_BP29[1]	GTTTATGCAAAGCAACATGG	GTTTATGCAAAGCAACATGG	51	1.5	1233
GCFC_29TO30	CACTTCACTTTCAATGTTGG	TAGCTGTTTAGCTCTCACCTG	55	1.5	591
GCFC_BP30	CTGTTCTTGCTTGGATATTAAG	CTCTTATGGGATTACTGGATTC	57	1.5	944
GCFC_BP31	TCCTGAAAGTTATTGGAAATG	GATCTTTTCATCTGTACTCCTG	54	1.5	810
GCFC_BP32	GGTTAGCCCACCAAATAACT	AAACTCGGAAATGAGACCTT	60	1.5	923
GCFC_BP33	GCCTGTGCTTTATCCATTTC	GAGCTCAGTTTGCAATCACA	60	1.5	931
GCFC_BP34	ATATCTTGGTCTTTGCTTTGC	AACCATACAGGACTAAGGACG	65	1.5	979
GCFC_BP35	CTCAGGCATCTAGCTCTTGG	TGCTCTATCGTTCACAAGGA	51.2	1.5	931
GCFC_BP36	TTAAATGTGTTATAAAGCCACAAAG	AAATATGCCCAAAGAAGAATAAGAC	59.8	1.5	907
GCFC_36TO37	GCCATTCAAGTCAGTCCATC	CAACAGCATTTGTGTTAGCG	55	1.5	383
GCFC_BP37	TAGCTTCACAAAGGAGTGCAAATC	GCCATGTCACCGTCTCTTTATG	67.8	1.5	967
GCFC_BP38	CTGGAAAGCATTCTCCTGTGTG	AAAGCGCTGGTGTCTACTTCAGT	66.4	1.5	1043
GCFC_38TO39	AGCTATCAGTTGAGTTGTCTGC	GTAGGGTCTCTTCTGCCATC	66.6	1.5	942
GCFC_BP39	AGAAGAAGTAGGATCTAGGGTAGTTAGG	GAATGATATGAAATGACAGCAAAGA	62	1.5	1007
GCFC_BP40	TTTCATACTTCCTACTTAGCAATCAC	GAATTCATCACGCTATTCAGTAATAA	58	1.5	901
GCFC_BP41	TGTAGTTGTTACCCTGAAATAATGTG	GAGAAACAAAATAAGCTTCTCCAAC	56.4	1.5	486
GCFC_BP42	TGCAGTTATCTGTCATTTATTAACTTTG	GATGCTAGGATCTTTGAGTGAAGTT	61	1.5	877
GCFC_BP43	TCCACTGTTCAGAGTCATATAGAGC	AAGAATGGTAAGATTACACCCAGTG	59.8	1.5	939
GCFC_43TO44	TTGAAGATGTCCTTGAAAGTT	CCTAGACAATTACCTCCCTTAG	55	1.5	613
GCFC_BP44	TACCTCATGCTCAGGGCTATAAGTG	AGGAAGAATCACCTTTTCAACAATG	58	1.5	1131
GCFC_BP45	ATATCGTAGCTGTGCCCATCCT	CGGACCCTGATGTTCTTATTCA	64.8	1.5	1040
GCFC_BP46	TTGTAAAGCACATTCACACTGAG	CACCAGTTAAGCAAATGGAAAC	56.4	1.5	1036
GCFC_BP47	CTCTTAGCCTTAACACCAGATAGC	AGCAAGGTTACATATAGTTCCACTG	53.5	1.5	895
GCFC_BP48	AACAATGCTTTAACAAGCGAAC	ACAACAACGGCTCTTAACAAAC	56.4	1.5	845
GCFC_BP49	ACCTCTTCCAAAACTTTCCCAC	CAAGTGAAAAGAGAAATATTCAGCC	62.6	1.5	762
GCFC_BP50	ACATTCTTTGAATTGTTTTGCTG	ATCAAAGGGGAAATAAGACATACA	58	1.5	1725
GCFC_BP51	ATGTACCATATAGGGAAATGACACT	CCAGACTGTGACCTTCTCTGTA	59.8	1.5	589
GCFC_BP52	ATTCAGACAGAGACCCACATACA	CAACATTCCAGTGCAACTTCTA	64.8	1.5	961

Table A.4. Continued

Primer	Forward	Reverse	Temperature	$MgCl_2$	Length
GCFC_intBP52	GATGAGATGGTTGGACTGAATC				
GCFC_BP53	AACTCCAAGATGCAGTCCTTATG	AGACATTGTTCTCTTTCCTTCTTG	48.5	3	993
GCFC_BP54	CTGTTCTCTTTGAGAAGGTATCTGC	GACCAACTGAAAAGTAGTGAAAGC	59.8	1.5	1007
GCFC_intBP54	GTTCCCTGTCCTTCACTATCTCC				
GCFC_BP55	CCTAGACTATTAGATTTTGCATGCC	GACCATTTTGAGAGTCTTTCTTG	59.8	1.5	1145
GCFC_BP56	AATGAAGCGTAAAACAGAAGCA	TTCAAGCTCAAGATTTCATACACA	59.8	1.5	946
GCFC_BP57	AAATGTTACAGAGGACACTCTCAGC	TAAGCCGCTCTCTATAGCAGCA	68	1.5	796
GCFC_BP58	AACACCTAAACCCAGAAGGCAG	GGAGACTCAGAAAATGCAGAGC	65	1.5	1011
GCFC_BP59int		AAAGACAGCCTTCAGAATGG			
GCFC_BP60	GAAGTTTTACCAAGGAAGATGC	AGGACTATACAACCAATACAGAAGC	52.5	1.5	1138
GCFC_BP61	AACTGTCTTCGCTAAATCAAGG	CTACTAAATTGTGACTCTGCATGG	65	1.5	568
GCFC_BP62[1]	CTACATTTGGTAATCATGTCTCAGG	CATTGTTTGAATCAGTTCAGTGC	63	1.5	407
GCFC_BP63	AAGAAAGACTCTCAAAATGGTCC	AAGCTCAAGATTTCATACACAACTG	65	1.5	904
GCFC_BP64[2]	AATCTCAAGTGAAATTTTGACAGAATGGTGTAGG	CTGACAGGCGGATCTATCTCAGTCTTTAGTG	66		5715
GCFC_intBP64.1	TATTTGAAAGTTGCTAAGAGAATGG	CATGAGTAGGATGAATTGGTATTGA			514
GCFC_intBP64.2	ATAGAGAGCGGCTTAGAATTGGC	TTTGTCCACCTGATTTGAAGAGC			588
GCFC_intBP64.3	AGTAATGCCATCCAACCACCTC	CTCAGATACGCAGATGACACCAC			665
GCFC_intBP64.4	TTCACTCTCCTCTTTCACCTTCATC	CACTCAGTACACCAGGAAATGTGG			692
GCFC_intBP64.5	CTTTGCTAGAATGTGAAATGAGTGC	AAGAAGCCTTACAAATAGCAGGAGA			623
GCFC_intBP64.6	TCCTCTATTTCTTTGCATTGTTCAC	GATTCTCCAGGCAAGAACACTG			804
GCFC_intBP64.7	CATGACTGAGCGACTTCACTTTC	GCCATAAGGGTGGTGTCATCTAC			689
GCFC_intBP64.8	AAGGCTGTATATTGTCACCCTGCT	TCACTCAGACTCATGTCCATCG			800
GCFC_intBP64.9	GAAGGAATGATGCTAAAGCTGAAAC	GTTGGAATGCAAAAGTAGGAAGTCA			797
GCFC794_1083	TCTGGATCATGCTATGTCTG	TACACTGGGAATGGTATTAGAG	55	3	124
GCFC637_792	AGCAGTGGTTCGTGAATCTT	CACATCAGAGCACCCAATAC	55	2	119
GCFC77_223	TGAGAACATGCTGTGGTTTG	TTCAACAATGGTAGGCAACA	55	3	103
GCFC488_638	TTCAGGAATTATCAATAGACGG	TTGATGCTGTCATCTCCATA	55	3	85
GCF2.1	AGRATGAGAAGAACTTTAGATGA	CAGAATTTYTATTTTCTAAGAC	50	2	448
GCF2.4	GATGTCGATGTCGCACTGTT	CTGCATTCACCACTGAAGGA	57	1.5	825

Table A.4. Continued

Primer	Forward	Reverse	Temperature	$MgCl_2$	Length
GCFC_WLK1	ATTCTCCAAGTGTATGTTCAGG				
GCFC_WLK2	TGCAAGCAAGCAACAAGTG				
GCFC_WLK3	CACCTTATACCAAACTCATTCC				
GCFC_WLK4	TAGGAGATGGTTTGAGAACG				
GCFC_WLK5	CTCAAACATTCCAAATCATTAC				
GCFC_WLK6	TGGTCAGGAGAGCAGAGCAG				
GCFC_WLK7	AACAAAACAATTCATCTTAACG				
GCFC_WLK8	TGACCTGTACCATGTCAGAG				
GCFC_WLK9	GTCAGTAGCAAATTGTCTTAGG				
GCFC_WLK10	CCCAAATTCCAGTAAGACAC				
GCFC_WLK11	AGTTCATGCCAATATTCTTGC				
GCFC_WLK12	GGAAAGGCAAAACAGTGAGA				
GCFC_WLK13	TTGAGAAGCTCTGCAATTAC				
GCFC_WLK14	TTGCACTACTAAGAAATCCATC				
GCFC_WLK15	AGTCATTCTCAGCAATCCTC				
GCFC_WLK16	TAACTTGCTTATCTCTGTTTCC				
GCFC_SEQ1	AATCCCGAAACATGTAATAGC				
GCFC_SEQ2	ATATTCACCATTTGAAGACAGG				

[1]Primer designed in a repetitive region. BAC DNA used to generate a single PCR product
[2]Non-standard PCR conditions

Table A.5. Gene specific PCR primers used to test for qualitative gene expression

Gene	Forward Sequence	Reverse Sequence	Expected Size gDNA	Expected Size cDNA	T_m
TIAM1	CGCAGAAAGTCAAAGTGTCG	CCCAAGATCTCTTCGTTGCT	594	594	58
LOC150051	TCTCCTCTTTGAGTCAGGCA	TCTGCATAGGTGGAGGTGG	238	238	60
SFRS15	AGTTCGTGTATTGAACCTTTG	GGTACAGTTGGTACAGAGTTTG	522	201	58
HUNK	TTGATGGAACATGCCAGAAC	TCTGAATCTGCGTGATGAGC	373	373	57
C21orf45	CGGATTGTTGTGAAGGATGT	ACAGCGTAGCAAGATGCAGT	323	323	58
MRAP	CATCTGGAGGCAACCCAG	CTCCCATCGAGAGGTTCTGT	234	234	58
C21orf63	TGACCCATCAGTTGCTAATC	GCTGACAATGACTCCATCTT	1825	115	55
C21orf59	CCATCATTAGCAGCGAGGAG	GGCAAACTTCATCTGGGTCT	425	187	58
SYNJ1	AGTTGCAATACGAATGCTGT	TGCTGTCTGATAAGCTCTTTC	470	234	64
GCF2.4	GATGTCGATGTCGCACTGTT	CTGCATTCACCACTGAAGGA	825	166	55
C21orf62	AACAGAGTTTGCTCATCCAC	GGCACAGCTACGATATTAGTAA	263	263	55
LOC440778	CTTCCAGTCCCTTCTACACC	TAAGCCAGCTCTCCTGACTC	664	664	60
OLIG2	CGCAAGCGCATGCACGACCTYAA	CRCAGGCCGACGGGTGGAARCC	217	217	62
OLIG1	GGCGCAAGATCAACAGC	CCAGCAGCAGGATGTAGTTG	174	174	60
IFNAR2	CTGTCAGTCTGGGTGTTTGC	CGTTAGTCCATGCTTGGTGT	177	177	55
IL10RB	TGATGAGAGTTCAGAGTGGA	CATGTAAAGAATTAGCAAGTGC	1767	104	56
IFNAR1	GTGGTCATTTATGTTGTGAGC	GAAGTGGAAAGGAGTAGATTCC	1357	131	55
SOX9	TGCTCAAGGGCTACGACTG	CGAGATGTGCGTCTGTTCC	788	358	64
COL2A1	CTGGAACTGCTGGTGCTC	TTCTCCTTGTTCACCTTTGG	691	125	55
COL9A2.1	ATAGAGGACGTGGTGCTGAAGA	TTTGCCGTTGATTGCCTG	1843	336	55
AGC1	GAGGGAAGGTTGCTATGGAG	CTGCCTCTTGGAAGGTGAAC	435	161	64
CDRAP	GTTTCTTGACCATACACCAGG	TCACGTACGATGCTACTGGG	619	151	59
SOX5	TGAAGTTGATGGCAATAAAG	CTTCCATTTTCCTCTGTTTC	188	188	53
SOX6	AACTACCACACCGTCACCTC	GGCCTTTTGAATATGCTCTG	750	173	58
PTHLH	TTGCCTGTGTGGTGTGGAA	CACCGAGTAGCTCAGCAGGA	306	134	60
IHH	CGTGCTCATTTTCCTCGATC	TGTCCCATGCCTCGTTAATG	286	286	64
PRKCA	CCTGAAGCCAGAGAACCTCC	GCCATTTCGTAGATGAGGAC	311	178	56
FGFR3	CTAACACCACCGACAAGGAG	CCAGGATGAAGAGGAGGAAG	687	225	60
STAT1	GTCAGCCAGCTCCCAAGTG	CCTGCACAAGGTGGGTTC	827	101	62
MAPK1	CATCGACATCTGGTCCGTC	AATTCAGGTCTTCCTGCGAC	392	137	64
TWIST1	AGCAGAACCCAAATTCAAAGAAAC	TGCCCGTCTGGGAATCAC	92	92	58
TWIST2	CGACGAGATGGACAATAAGATGAC	CACACGGAGAAGGCGTAGCT	75	75	58
RUNX1	GGGAGCTGCTTGCTGAAGAT	ACAACAAGCCGATTGAGTTAGGA	82	82	58
RUNX2	CCAACCCACGAATGCACTATC	AGGGACATGCCTGAGGTGACT	66	66	58
COL18A1	GATCCATGGGCACTTCTTCAG	TCCACCAGCATCTGGTACGT	61	61	63
BMP4	AACACCGTGAGGAGCTTCCA	TGCTGAGGTTAAAGAGGAAACGA	1024	84	58
PISRT1	TGTTAGCTTTGCTTACAAGGATGTTC	GGTCAGAGGAAGCAGAAATGAAA	75	75	58
ACTB	CATCCTGACCCTCAAGTACCC	GTGGTGGTGAAGCTGTAGCC	420	860	58

Table A.6. Gene specific PCR primers used in real-time PCR

Primer	Forward	Reverse	Size (bp)
BMP4_quant	AACACCGTGAGGAGCTTCCA	TGCTGAGGTTAAAGAGGAAACGA	94
C21ORF45_quant	GAGCTGGGTGGCGAGTCA	TGTTCCTCATTCACAGAAACATTACA	80
C21ORF59_quant	GGCGCAGCGCATCTG	TCATCAGTCAGTCCTTGCATATTAGG	83
C21ORF62_quant	TGGGCACAGCTTCTTTCTGA	CCTCTGAGCTCTGGTGAAGCA	69
C21ORF66_quant	CATGCCAAACGTCGGATTG	CAAGGTGATCTGCCATCTTACCA	68
IL10RB_quant	CACCTTCTGTCCTGTGGATGAC	AACGCACATGTAAAGAATTAGCAAGT	57
SFRS15_quant	CACCTCCACCTCCAGTTAAAGTTT	TGGGCAACTGCGGTACAGT	72
SYNJ1_quant2	GAATGCTGTTCCACACCACAA	TTTCGTTCTTTGACTTGGGATTG	54
COL18A1_quant	GATCCATGGGCACTTCTTCAG	TCCACCAGCATCTGGTACGT	61
FOXL2_quant	CTCACGCTGTCCGGCATCT	CGTAGAACGGGAACTTGGCTATAAT	52
PTHLH_quant	AACATCGCTGGAGCTCAACTC	CCTGCAATACGTCTTCTGAAGGT	70
PRKACA_quant	GGACCTGAAGCCAGAGAACCT	TTCACACGCTTGGCGAAC	80
RUNX1_quant	GGGAGCTGCTTGCTGAAGAT	ACAACAAGCCGATTGAGTTAGGA	82
RUNX2_quant	CCAACCCACGAATGCACTATC	AGGGACATGCCTGAGGTGACT	66
SOX9_quant	AATCTCCTGGACCCCTTCATG	GGCGGACAGGCCCTTCT	57
STAT1_quant	GCCTCTATCCTGTGGTACAACATG	ACAAGGTGGGTTCAGGAAGAAG	68
TWIST1_quant	AGCAGAACCCAAATTCAAAGAAAC	TGCCCGTCTGGGAATCAC	92
TWIST2_quant	CGACGAGATGGACAATAAGATGAC	CACACGGAGAAGGCGTAGCT	75
CDRAP_quant	AAGCTGGCTGACCGGAAGAT	GCCACAGCCACGGAGATG	64
TIAM1_quant	TTTCGACACATGATTCCTACTGAAG	GACGATTTCACACACAGCATTTG	82
18S_quant[1]	AGAAACGGCTACCACATCCA	CACCAGACTTGCCCTCCA	169
GAPD_quant[1]	TTCAACGGCACAGTCAAGG	ACATACTCAGCACCAGCATCAC	119
B-actin_quant[2]	CGCCATGGATGATGATATTGC	AAGCCGGCCTTGCACAT	65
YWHAZ_quant[1]	GCATCCCACAGACTATTT	GCAAAGACAATGACAGACCA	120
SDHA_quant[1]	GCAGAACCTGATGCTTTGTG	CGTAGGAGAGCGTGTGCTT	185

[1]Goosens et al., 2005.
[2]Graphodatskaya et al., 2006

Table A.7. Samples from 1 to 8 d old calves used in real-time PCR

Animal	Sex	Breed[1]	Type[2,3]	Birth Date	Date Collected	RIN
022T	Male	Angus x Brahman	Putative Scur	2/19/2007	2/26/2007	6.7
037T	Male	Angus	Poll	2/20/2007	2/26/2007	6.8
053T	Male	Angus x Brahman	Putative Scur	2/21/2007	2/26/2007	7
092T	Male	Angus	Poll	2/26/2007	2/26/2007	7.2
093T	Female	Angus	Poll	2/26/2007	2/26/2007	7.2
096T	Female	Angus	Poll	2/26/2007	2/26/2007	7.8
097T	Male	Angus	Poll	2/26/2007	2/26/2007	6.3
098T	Female	Angus	Poll	2/26/2007	2/26/2007	7.1
104T	Female	BA x NA	Horn	2/26/2007	2/26/2007	7.5
106T	Male	BH x NA	Horn	2/26/2007	2/26/2007	7.1
229T	Male	Nellore x Angus	Putative Scur	3/7/2007	3/7/2007	6.8
230T	Male	Nellore x Angus	Putative Scur	3/7/2007	3/7/2007	7.6
231T	Female	Nellore x Angus	Putative Scur	3/7/2007	3/7/2007	7.6
234T	Female	Angus x Nellore	Putative Scur	3/7/2007	3/7/2007	7.4
235T	Female	Angus x Brahman	Putative Scur	3/7/2007	3/7/2007	7.5
239T	Male	HB x NA	Horn	3/7/2007	3/7/2007	6
243T	Male	HB x NA	Horn	3/7/2007	3/7/2007	7.6
246T	Male	BA x NA	Horn	3/7/2007	3/7/2007	6.7
364T	Female	BA x NA	Horn	3/19/2007	3/20/2007	7.9
924T	Female	Charolais x BANH	Horn	3/28/2007	4/5/2007	8.2

[1]B = Brahman, A = Angus, N = Nellore, H = horned Hereford

[2]Poll = skin sampled from the same location as horn or scurs were collected

[3]Based on breed type and observation at time of sample collection; samples that were homozygous *Bos indicus* (horned) were identified by genotype.

Table A.8. Samples from 5 to 6 mo old calves used in real-time RT-PCR

Animal	Sex	Breed	Type[1,2]	Birth Date	Date Collected	RIN[3]
358S	Male	Angus x Brahman	Scur	3/13/2006	9/19/2006	5.5
414S	Male	Nellore x Angus	Scur	3/20/2006	9/13/2006	6.5
416S	Female	Angus	Poll	3/21/2006	9/13/2006	7.7
433S	Female	Angus	Poll	3/22/2006	9/13/2006	7.2
438S	Male	Angus x Brahman	Scur	3/23/2006	9/19/2006	7.5
449S	Female	Angus x Brahman	Scur	3/28/2006	9/19/2006	5.2
450S	Male	Angus x Brahman	Scur	3/28/2006	9/19/2006	6
729S	Male	Angus	Poll	3/28/2006	9/13/2006	7.6
739S	Female	Angus x Brahman	Scur	3/30/2006	9/19/2006	6.6
751S	Male	Angus	Poll	3/30/2006	9/13/2006	7.8
761S	Female	Nellore	Horn	4/4/2006	9/14/2006	5.9
763S	Female	Nellore	Horn	4/4/2006	9/14/2006	5.8
777S	Male	Angus	Poll	4/12/2006	9/13/2006	7
778S	Female	Angus	Poll	4/12/2006	9/13/2006	7.5
780S	Male	Nellore	Horn	4/12/2006	9/14/2006	6
781S	Female	Nellore	Horn	4/12/2006	9/14/2006	6.4
786S	Male	Nellore	Horn	4/20/2006	9/14/2006	6
787S	Male	Nellore	Horn	4/20/2006	9/14/2006	6.5

[1]Based on breed type and observation at time of sample collection
[2]Poll = skin sampled from the same location as horn or scurs were collected
[3]RIN = RNA integrity number

Table A.9. Relative levels of expression of genes from the polled interval

Age[1]	Type[2,3]	*C21orf45*	*C21orf59*	*C21orf62*	*C21orf66*	*IL10RB*	*SFRS15*	*SYNJ1*
1 to 8 d	Horn	2.8 +/- 0.3	1.4 +/- 0.1	1.5 +/- 0.3	1.5 +/- 0.2	2.0 +/- 0.3	1.2 +/- 0.1	0.9 +/- 0.1
	Scur	1.9 +/- 0.3	1.3 +/- 0.1	2.5 +/- 0.5	1.7 +/- 0.1	1.6 +/- 0.2	1.2 +/- 0.0	1.1 +/- 0.1
	Poll	2.5 +/- 0.4	1.4 +/- 0.1	1.1 +/- 0.2	1.2 +/- 0.2	1.5 +/- 0.2	1.0 +/- 0.2	1.2 +/- 0.2
5 to 6 mo	Horn	0.9 +/- 0.2	0.8 +/- 0.1	1.2 +/- 0.2	1.1 +/- 0.1	0.7 +/- 0.1	1.0 +/- 0.1	0.6 +/- 0.0
	Scur	0.8 +/- 0.2	0.7 +/- 0.1	0.7 +/- 0.1	0.8 +/- 0.1	0.9 +/- 0.1	0.8 +/- 0.1	0.6 +/- 0.1
	Poll	1.2 +/- 0.3	1.0 +/- 0.0	1.0 +/- 0.3	1.0 +/- 0.1	1.1 +/- 0.1	0.9 +/- 0.1	0.8 +/- 0.1

[1]Data within each age group are relative, but data are not relative across age groups because separate calibrators were used.
[2]Based on breed type and observation at time of sample collection, remaining scur from 1 to 8 d old calves was observed again in fields again at ~6 mos, 1 to 8 day old horns were verified by genotyping.
[3]Poll = skin sampled from the same location as horn or scurs were collected

Table A.10. Relative levels of expression of genes involved in chondrogenesis and osteogenesis

Age[1]	Type[2,3]	*BMP4*	*COL18A1*	*FOXL2*	*PTHLH*	*PRKCA*	*RUNX1*	*RUNX2*	*SOX9*	*STAT1*	*TWIST1*	*TWIST2*
1 to 8 d	Horn	0.9 +/- 0.1	2.0 +/- 0.2	1.2 +/- 0.2	1.4 +/- 0.2	0.9 +/- 0.0	2.6 +/- 0.4	5.6 +/- 0.7	0.8 +/- 0.1	1.4 +/- 0.1	1.6 +/- 0.1	1.8 +/- 0.1
	Scur	1.5 +/- 0.1	2.1 +/- 0.4	1.3 +/- 0.2	1.8 +/- 0.2	1.1 +/- 0.1	2.8 +/- 0.4	4.5 +/- 1.5	1.1 +/- 0.2	1.5 +/- 0.3	2.0 +/- 0.3	2.0 +/- 0.3
	Poll	1.3 +/- 0.2	2.0 +/- 0.3	2.2 +/- 0.5	2.0 +/- 0.6	1.2 +/- 0.0	1.9 +/- 0.3	1.0 +/- 0.1	2.0 +/- 0.4	1.2 +/- 0.1	1.4 +/- 0.1	1.4 +/- 0.1
5 to 6 mo	Horn	0.4 +/- 0.1	4.7 +/- 1.23	2.0 +/- 0.4	0.6 +/- 0.1	0.5 +/- 0.0	1.2 +/- 0.1	2.6 +/- 0.4	0.6 +/- 0.1	0.8 +/- 0.1	0.9 +/- 0.1	0.7 +/- 0.1
	Scur	0.4 +/- 0.0	8.8 +/- 4.0	1.0 +/- 0.2	1.0 +/- 0.1	0.6 +/- 0.0	1.2 +/- 0.1	1.7 +/- 0.3	0.6 +/- 0.2	0.8 +/- 0.1	0.6 +/- 0.1	0.7 +/- 0.1
	Poll	0.8 +/- 0.1	5.1 +/- 2.0	1.4 +/- 0.4	1.0 +/- 0.1	0.8 +/- 0.1	1.1 +/- 0.1	2.2 +/- 0.9	0.9 +/- 0.1	1.0 +/- 0.1	1.0 +/- 0.1	1.0 +/- 0.0

[1]Data within each age group are relative, but data are not relative across age groups because separate calibrators were used.
[2]Based on breed type and observation at time of sample collection, remaining scur from 1 to 8 d old calves was observed again in fields again at ~6 mos, 1 to 8 day old horns were verified by genotyping.
[3]Poll = skin sampled from the same location as horn or scurs were collected

Table A.11. Relative levels of expression of genes located in the polled interval by sex of calf

Age[1]	Type[2,3]	Sex[4]	*C21orf45*	*C21orf59*	*C21orf62*	*C21orf66*	*IL10RB*	*SFRS15*	*SYNJ1*
1 to 8 d	Horn	Female	2.6 +/- 0.4	1.3 +/- 0.2	1.6 +/- 0.6	1.2 +/- 0.4	1.6 +/- 0.1	1.2 +/- 0.2	0.8 +/- 0.1
		Male	2.9 +/- 0.5	1.5 +/- 0.2	1.5 +/- 0.4	1.8 +/- 0.1	2.4 +/- 0.6	1.1 +/- 0.2	1.1 +/- 0.1
	Scur	Female	1.7 +/- 0.4	1.3 +/- 0.2	2.2 +/- 0.5	1.9 +/- 0.1	1.7 +/- 0.2	1.2 +/- 0.1	1.2 +/- 0.1
		Male	2.1 +/- 0.6	1.4 +/- 0.1	2.8 +/- 0.9	1.5 +/- 0.1	1.3 +/- 0.3	1.3 +/- 0.0	1.0 +/- 0.2
	Poll	Female	2.5 +/- 0.7	1.5 +/- 0.2	1.5 +/- 0.2	1.5 +/- 0.1	1.4 +/- 0.2	1.2 +/- 0.3	1.0 +/- 0.1
		Male	2.4 +/- 0.7	1.3 +/- 0.2	0.8 +/- 0.2	1.0 +/- 0.2	1.6 +/- 0.3	0.8 +/- 0.1	1.4 +/- 0.3
5 to 6 mo	Horn	Female	0.9 +/- 0.3	0.8 +/- 0.1	1.3 +/- 0.2	1.1 +/- 0.0	0.8 +/- 0.2	1.1 +/- 0.1	0.5 +/- 0.1
		Male	0.9 +/- 0.4	0.7 +/- 0.1	1.2 +/- 0.5	1.0 +/- 0.1	0.7 +/- 0.0	0.9 +/- 0.1	0.6 +/- 0.0
	Scur	Female	1.1 +/- 0.4	0.8 +/- 0.3	0.7 +/- 0.2	0.8 +/- 0.1	0.6 +/- 0.1	0.8 +/- 0.1	0.7 +/- 0.1
		Male	0.6 +/- 0.4	0.7 +/- 0.1	0.7 +/- 0.2	0.8 +/- 0.1	1.1 +/- 0.1	0.8 +/- 0.1	0.5 +/- 0.1
	Poll	Female	1.4 +/- 0.7	1.0 +/- 0.0	1.1 +/- 0.6	1.0 +/- 0.2	1.1 +/- 0.1	0.8 +/- 0.1	0.7 +/- 0.1
		Male	1.0 +/- 0.2	1.0 +/- 0.0	0.9 +/- 0.3	1.0 +/- 0.1	1.0 +/- 0.1	1.0 +/- 0.1	0.9 +/- 0.1

[1]Data within each age group are relative, but data are not relative across age groups because separate calibrators were used.
[2]Based on breed type and observation at time of sample collection, remaining scur from 1 to 8 d old calves was observed again in fields again at ~6 mos, 1 to 8 day old horns were verified by genotyping.
[3]Poll = skin sampled from the same location as horn or scurs were collected
[4]Males (5 to 6 mo) were castrated at 7 to 30 d old; 414S and 787S were left intact.

Table A.12. Relative levels of expression of genes involved in chondrogenesis and osteogenesis by sex of calf

Age[1]	Type[2,3]	Sex[4]	*BMP4*	*COL18A1*	*FOXL2*	*PTHLH*	*PRKACA*	*RUNX1*	*RUNX2*	*SOX9*	*STAT1*	*TWIST1*	*TWIST2*
1 to 8 d	Horn	Female	1.0 +/- 0.3	1.8 +/- 0.1	0.9 +/- 0.2	1.4 +/- 0.4	0.9 +/- 0.0	2.3 +/- 0.8	4.5 +/- 1.0	0.8 +/- 0.2	1.3 +/- 0.1	1.4 +/- 0.1	1.8 +/- 0.2
		Male	0.9 +/- 0.1	2.2 +/- 0.3	1.4 +/- 0.1	1.4 +/- 0.1	0.9 +/- 0.1	2.9 +/- 0.3	6.6 +/- 0.6	0.9 +/- 0.1	1.6 +/- 0.1	1.9 +/- 0.2	1.7 +/- 0.2
	Scur	Female	1.6 +/- 0.1	1.9 +/- 0.3	1.1 +/- 0.2	1.7 +/- 0.2	1.2 +/- 0.0	2.6 +/- 0.4	3.5 +/- 0.8	1.0 +/- 0.1	1.4 +/- 0.2	1.9 +/- 0.2	1.9 +/- 0.2
		Male	1.4 +/- 0.1	2.3 +/- 0.8	1.5 +/- 0.4	1.8 +/- 0.3	1.0 +/- 0.2	2.9 +/- 0.7	5.4 +/- 3.1	1.2 +/- 0.4	1.7 +/- 0.6	2.1 +/- 0.5	2.1 +/- 0.6
	Poll	Female	1.5 +/- 0.2	2.1 +/- 0.4	1.9 +/- 0.8	2.7 +/- 1.2	1.2 +/- 0.0	2.1 +/- 0.4	1.1 +/- 0.3	1.9 +/- 0.7	1.2 +/- 0.2	1.5 +/- 0.1	1.5 +/- 0.3
		Male	1.1 +/- 0.1	1.8 +/- 0.5	2.4 +/- 0.7	1.4 +/- 0.4	1.1 +/- 0.1	1.6 +/- 0.3	0.9 +/- 0.1	2.2 +/- 0.6	1.2 +/- 0.1	1.3 +/- 0.2	1.4 +/- 0.2
5 to 6 mo	Horn	Female	0.4 +/- 0.1	6.6 +/- 2.1	1.7 +/- 0.6	0.5 +/- 0.1	0.5 +/- 0.1	1.2 +/- 0.2	2.8 +/- 0.8	0.7 +/- 0.2	0.7 +/- 0.1	0.9 +/- 0.2	0.7 +/- 0.2
		Male	0.3 +/- 0.1	2.8 +/- 0.4	2.2 +/- 0.6	0.7 +/- 0.2	0.5 +/- 0.0	1.1 +/- 0.2	2.4 +/- 0.2	0.6 +/- 0.2	0.9 +/- 0.2	1.0 +/- 0.3	0.8 +/- 0.2
	Scur	Female	0.4 +/- 0.0	7.3 +/- 5.2	1.2 +/- 0.3	1.0 +/- 0.2	0.6 +/- 0.0	1.4 +/- 0.3	1.9 +/- 0.6	0.7 +/- 0.3	0.9 +/- 0.2	0.7 +/- 0.3	0.7 +/- 0.2
		Male	0.3 +/- 0.1	10.3 +/- 7.1	0.9 +/- 0.3	1.0 +/- 0.2	0.6 +/- 0.1	1.1 +/- 0.1	1.4 +/- 0.4	0.5 +/- 0.1	0.7 +/- 0.1	0.5 +/- 0.1	0.7 +/- 0.1
	Poll	Female	0.7 +/- 0.1	8.0 +/- 3.4	1.4 +/- 0.5	1.0 +/- 0.1	0.7 +/- 0.1	1.1 +/- 0.1	1.5 +/- 0.4	0.9 +/- 0.2	0.9 +/- 0.3	0.9 +/- 0.1	1.0 +/- 0.1
		Male	0.9 +/- 0.1	2.2 +/- 0.6	1.5 +/- 0.7	0.9 +/- 0.1	0.9 +/- 0.1	1.1 +/- 0.2	2.9 +/- 1.8	0.8 +/- 0.1	1.0 +/- 0.1	1.1 +/- 0.1	1.0 +/- 0.0

[1]Data within each age group are relative, but data are not relative across age groups because separate calibrators were used.
[2]Based on breed type and observation at time of sample collection, remaining scur from 1 to 8 d old calves was observed again in fields again at ~6 mos, 1 to 8 day old horns were verified by genotyping.
[3]Poll = skin sampled from the same location as horn or scurs were collected
[4]Males (5 to 6 mo) were castrated at 7 to 30 d old; 414S and 787S were left intact.

Table A.13. Relative levels of expression in samples from 1 to 8 d old calves by animal

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
BMP4	Horn	Female	104T	29.04	0.13	1.0
			364T	28.68	0.08	1.4
			924T	29.74	0.14	0.5
		Male	106T	30.11	0.38	1.1
			239T	30.47	0.04	0.9
			243T	31.53	0.20	0.8
	Poll	Female	093T	30.48	0.29	1.7
			096T	29.32	0.10	1.8
			098T	30.05	0.13	1.1
		Male	037T	31.65	0.24	0.9
			092T	31.74	0.26	1.4
			097T	30.11	0.07	1.0
	Scur	Female	231T	29.91	0.03	1.7
			234T	28.87	0.08	1.8
			235T	29.50	0.17	1.3
		Male	053T	29.34	0.02	1.4
			229T	31.90	0.05	1.6
			230T	29.40	0.03	1.2
C21orf45	Horn	Female	104T	27.19	0.14	3.2
			364T	27.56	0.08	2.0
			924T	27.12	0.07	2.6
		Male	106T	28.03	0.02	2.5
			239T	28.28	0.14	2.4
			243T	26.88	0.14	3.8
	Poll	Female	093T	27.16	0.02	3.8
			096T	27.99	0.12	1.9
			098T	27.85	0.12	1.8
		Male	037T	27.66	0.10	3.0
			092T	28.09	0.10	3.3
			097T	28.87	0.10	1.0
	Scur	Female	231T	28.19	0.12	1.4
			234T	28.32	0.06	1.3
			235T	27.77	0.03	2.4
		Male	053T	28.80	0.12	1.1
			229T	28.33	0.19	3.2
			230T	27.66	0.09	1.9

Table A.13. Continued

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
C21orf59	Horn	Female	104T	27.68	0.12	1.4
			364T	27.63	0.11	1.6
			924T	27.83	0.07	1.0
		Male	106T	28.20	0.13	1.1
			243T	27.87	0.02	1.6
			246T	31.85	0.01	1.8
	Poll	Female	096T	27.82	0.05	1.4
			093T	28.84	0.41	1.9
			098T	28.10	0.12	1.1
		Male	037T	29.68	0.11	1.3
			092T	29.54	0.21	1.6
			097T	28.23	0.05	1.0
	Scur	Female	231T	28.02	0.49	1.1
			234T	27.72	0.11	1.1
			235T	27.73	0.13	1.8
		Male	022T	40.00	0.00	NA
			063T	28.46	0.05	1.3
			230T	27.27	0.07	1.5
C21orf62	Horn	Female	104T	33.74	0.10	0.7
			364T	32.61	0.16	1.5
			924T	30.97	0.25	2.6
		Male	106T	31.70	0.35	2.2
			239T	32.76	0.30	1.4
			243T	33.68	0.28	0.8
	Poll	Female	093T	32.36	0.16	1.5
			096T	32.91	0.00	1.1
			098T	31.63	0.45	2.0
		Male	037T	34.23	0.17	0.4
			092T	33.84	0.20	0.9
			097T	32.92	0.43	1.0
	Scur	Female	231T	32.01	0.39	2.0
			234T	31.22	0.32	3.1
			235T	32.78	0.58	1.4
		Male	053T	31.31	0.16	3.0
			229T	33.80	0.45	1.2
			230T	30.44	0.53	4.2

Table A.13. Continued

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
C21orf66	Horn	Female	104T	27.73	0.07	1.7
			364T	27.45	0.08	1.6
			924T	30.01	0.11	0.4
		Male	106T	27.87	0.07	1.9
			239T	28.74	0.08	1.7
			243T	28.35	0.02	1.7
	Poll	Female	093T	28.84	0.02	1.5
			096T	27.89	0.04	1.6
			098T	28.50	0.03	1.4
		Male	037T	30.86	0.05	0.5
			092T	30.14	0.14	1.4
			097T	28.24	0.05	1.0
	Scur	Female	231T	28.02	0.04	2.0
			234T	28.07	0.07	1.8
			235T	28.04	0.14	1.8
		Male	053T	28.56	0.03	1.6
			229T	30.77	0.04	1.4
			230T	28.09	0.03	1.5
COL18A1	Horn	Female	104T	26.15	0.02	1.6
			364T	25.63	0.04	1.8
			924T	26.04	0.04	2.1
		Male	106T	26.36	0.06	1.6
			239T	26.52	0.04	2.5
			243T	26.06	0.01	2.5
	Poll	Female	093T	26.09	0.12	3.0
			096T	26.08	0.05	1.6
			098T	26.55	0.03	1.7
		Male	037T	26.86	0.03	2.6
			092T	28.05	0.04	1.9
			097T	26.56	0.06	1.0
	Scur	Female	231T	26.76	0.05	1.4
			234T	26.32	0.00	1.9
			235T	26.10	0.04	2.4
		Male	053T	27.54	0.01	0.9
			229T	27.64	0.03	3.6
			230T	25.82	0.07	2.3

Table A.13. Continued

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
FOXL2	Horn	Female	104T	27.08	0.03	0.5
			364T	26.34	0.03	0.9
			924T	25.03	0.09	1.2
		Male	106T	25.19	0.04	1.5
			239T	25.89	0.04	1.3
			243T	25.79	0.06	1.5
	Poll	Female	093T	24.18	0.17	3.3
			096T	26.67	0.11	0.6
			098T	24.69	0.25	1.8
		Male	037T	24.36	0.21	3.2
			092T	24.96	0.18	3.1
			097T	25.88	0.10	1.0
	Scur	Female	231T	26.64	0.11	0.6
			234T	25.47	0.01	1.3
			235T	25.74	0.40	1.4
		Male	053T	25.99	0.06	0.9
			229T	25.87	0.06	2.2
			230T	25.16	0.07	1.3
IL10RB	Horn	Female	104T	28.20	0.03	1.4
			364T	28.10	0.01	1.7
			924T	27.69	0.05	1.6
		Male	106T	28.52	0.07	1.3
			243T	27.72	0.04	2.7
			246T	31.52	0.15	3.3
	Poll	Female	096T	28.50	0.03	1.3
			093T	29.51	0.09	1.7
			098T	28.48	0.08	1.2
		Male	037T	29.71	0.10	1.8
			092T	29.69	0.02	2.1
			097T	28.78	0.23	1.0
	Scur	Female	231T	27.78	0.09	1.9
			234T	27.99	0.07	1.3
			235T	28.15	0.11	2.0
		Male	022T	36.54	0.33	NA
			063T	29.40	0.63	1.0
			230T	27.67	0.04	1.6

Table A.13. Continued

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
PRKCA	Horn	Female	104T	26.90	0.07	0.9
			364T	26.61	0.04	0.9
			924T	27.20	0.05	0.9
		Male	106T	27.31	0.05	0.8
			239T	28.16	0.12	0.8
			243T	27.25	0.10	1.1
	Poll	Female	093T	27.40	0.06	1.2
			096T	26.49	0.10	1.2
			098T	27.01	0.04	1.2
		Male	037T	27.95	0.00	1.2
			092T	28.72	0.06	1.2
			097T	26.54	0.04	1.0
	Scur	Female	231T	27.01	0.02	1.2
			234T	27.05	0.12	1.1
			235T	27.09	0.03	1.2
		Male	053T	27.89	0.02	0.7
			229T	29.12	0.10	1.3
			230T	27.09	0.04	0.9
PTHLH	Horn	Female	104T	31.51	0.02	1.7
			364T	31.49	0.02	1.8
			924T	32.54	0.22	0.7
		Male	106T	33.25	0.20	1.2
			239T	32.95	0.00	1.5
			243T	33.74	0.32	1.5
	Poll	Female	093T	32.10	0.02	5.0
			096T	32.96	0.10	1.4
			098T	32.67	0.21	1.6
		Male	037T	34.64	0.07	1.1
			092T	34.30	0.30	2.1
			097T	33.33	0.09	1.0
	Scur	Female	231T	33.13	0.39	1.7
			234T	31.88	0.00	2.0
			235T	32.57	0.28	1.5
		Male	053T	32.39	0.12	1.6
			229T	34.54	0.24	2.5
			230T	32.49	0.07	1.3

Table A.13. Continued

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
RUNX1	Horn	Female	104T	26.39	1.29	1.3
			364T	25.66	0.04	1.7
			924T	25.08	0.07	3.9
		Male	106T	25.87	0.12	2.2
			239T	26.12	0.02	3.2
			243T	25.65	0.05	3.2
	Poll	Female	093T	26.05	0.05	3.0
			096T	25.96	0.01	1.7
			098T	26.56	0.03	1.6
		Male	037T	27.32	0.07	1.8
			092T	27.86	0.02	2.1
			097T	26.52	0.18	1.0
	Scur	Female	231T	26.07	0.12	2.3
			234T	26.12	0.05	2.1
			235T	25.54	0.02	3.5
		Male	053T	26.69	0.07	1.6
			229T	27.44	0.01	4.0
			230T	25.40	0.03	3.0
RUNX2	Horn	Female	104T	27.09	0.01	3.8
			364T	26.99	0.06	3.3
			924T	26.63	0.04	6.5
		Male	106T	26.41	0.10	7.4
			239T	27.25	0.01	7.0
			243T	27.17	0.02	5.4
	Poll	Female	093T	29.91	0.03	1.0
			096T	29.59	0.08	0.7
			098T	28.93	0.01	1.5
		Male	037T	30.80	0.01	0.8
			092T	31.16	0.03	1.0
			097T	28.80	0.03	1.0
	Scur	Female	231T	27.61	0.03	3.8
			234T	28.46	0.19	2.0
			235T	27.42	0.07	4.6
		Male	053T	29.37	0.09	1.2
			229T	28.21	0.06	11.4
			230T	27.41	0.03	3.6

Table A.13. Continued

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
SFRS15	Horn	Female	104T	26.54	0.03	1.3
			364T	26.43	0.04	1.6
			924T	26.97	0.04	0.8
		Male	106T	26.90	0.07	1.2
			243T	26.91	0.03	1.4
			246T	31.79	0.06	0.8
	Poll	Female	096T	26.99	0.02	1.1
			093T	27.80	0.04	1.7
			098T	27.29	0.11	0.8
		Male	037T	29.61	0.07	0.6
			092T	29.59	0.10	0.7
			097T	27.02	0.05	1.0
	Scur	Female	231T	26.51	0.03	1.3
			234T	26.36	0.02	1.2
			235T	27.21	0.06	1.1
		Male	022T	36.39	0.54	NA
			063T	27.25	0.02	1.3
			230T	26.32	0.02	1.2
SOX9	Horn	Female	104T	26.23	0.02	1.1
			364T	26.56	0.02	0.7
			924T	27.18	0.03	0.5
		Male	106T	27.64	0.02	0.6
			239T	28.15	0.06	1.0
			243T	27.12	0.04	1.1
	Poll	Female	093T	26.30	0.13	3.2
			096T	26.16	0.03	1.5
			098T	26.81	0.01	0.9
		Male	037T	27.01	0.04	2.4
			092T	27.56	0.05	3.1
			097T	26.53	0.03	1.0
	Scur	Female	231T	27.36	0.19	0.9
			234T	26.46	0.03	1.2
			235T	26.95	0.02	1.0
		Male	053T	27.49	0.05	0.7
			229T	28.08	0.01	2.1
			230T	26.63	0.08	0.9

Table A.13. Continued

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
STAT1	Horn	Female	104T	28.00	0.02	1.2
			364T	27.90	0.02	1.3
			924T	27.44	0.05	1.4
		Male	106T	28.92	0.02	1.4
			239T	28.88	0.04	1.5
			243T	29.50	0.05	1.7
	Poll	Female	093T	29.73	0.11	1.6
			096T	29.28	0.02	1.1
			098T	29.57	0.05	0.9
		Male	037T	30.56	0.01	1.1
			092T	30.78	0.09	1.5
			097T	29.29	0.01	1.0
	Scur	Female	231T	29.41	0.08	1.4
			234T	28.17	0.01	1.6
			235T	28.95	0.03	1.1
		Male	053T	29.14	0.03	0.9
			229T	30.31	0.09	2.8
			230T	28.47	0.07	1.3
SYNJ1	Horn	Female	104T	29.26	0.07	0.9
			364T	29.31	0.14	0.7
			924T	29.41	0.11	0.8
		Male	106T	29.88	0.03	0.9
			239T	30.58	0.21	1.3
			243T	29.92	0.08	1.0
	Poll	Female	093T	30.34	0.12	1.3
			096T	29.76	0.04	0.8
			098T	29.65	0.10	0.8
		Male	037T	30.75	0.07	1.2
			092T	30.89	0.10	2.1
			097T	29.27	0.04	1.0
	Scur	Female	231T	29.59	0.05	1.2
			234T	29.62	0.19	0.9
			235T	29.26	0.04	1.4
		Male	053T	30.36	0.03	0.6
			229T	31.33	0.16	1.4
			230T	29.05	0.07	1.1

Table A.13. Continued

Detector	Type[1,2]	Sex	Sample	Ct Avg[3]	Ct SD[4]	RQ[5]
TWIST1	Horn	Female	104T	27.94	0.10	1.2
			364T	27.30	0.04	1.5
			924T	27.96	0.01	1.5
		Male	106T	27.40	0.04	2.2
			239T	28.38	0.13	1.8
			243T	28.16	0.10	1.6
	Poll	Female	093T	28.40	0.00	1.7
			096T	27.93	0.02	1.3
			098T	28.10	0.11	1.6
		Male	037T	29.45	0.09	1.2
			092T	29.55	0.09	1.8
			097T	27.99	0.11	1.0
	Scur	Female	231T	28.06	0.05	1.7
			234T	27.74	0.09	1.9
			235T	27.50	0.06	2.2
		Male	053T	28.75	0.01	1.2
			229T	29.39	0.04	3.0
			230T	27.49	0.09	2.0
TWIST2	Horn	Female	104T	27.28	0.06	1.8
			364T	27.18	0.04	1.5
			924T	27.44	0.03	2.0
		Male	106T	28.01	0.01	1.4
			239T	28.18	0.03	1.9
			243T	27.83	0.04	1.9
	Poll	Female	093T	28.06	0.05	2.0
			096T	27.96	0.04	1.2
			098T	28.30	0.02	1.3
		Male	037T	28.98	0.02	1.6
			092T	29.68	0.12	1.5
			097T	27.90	0.03	1.0
	Scur	Female	231T	28.03	0.04	1.6
			234T	27.71	0.08	1.8
			235T	27.41	0.03	2.2
		Male	053T	28.50	0.03	1.3
			229T	29.21	0.10	3.2
			230T	27.52	0.07	1.8

[1]Based on breed type and observation at time of sample collection
[2]Poll = skin sampled from the same location as horn or scurs were collected
[3]Average cycle threshold value
[4]Standard deviation of cycle threshold
[5]Relative quantity

Table A.14. Relative levels of expression in samples 5 to 6 mo old calves by animal

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
BMP4	Horn	Female	761S	30.09	0.05	0.5
			763S	29.86	0.08	0.5
			781S	30.48	0.14	0.3
		Male	780S	30.89	0.08	0.5
			786S	30.58	0.10	0.2
			787S	33.46	0.35	0.2
	Poll	Female	416S	28.42	0.02	1.0
			433S	29.71	0.17	0.6
			778S	30.20	0.06	0.7
		Male	729S	29.34	0.10	0.9
			751S	28.13	0.09	1.0
			777S	28.72	0.10	0.8
	Scur	Female	438S	31.41	0.12	0.5
			449S	31.01	0.06	0.4
			739S	30.72	0.13	0.3
		Male	358S	33.17	0.32	0.4
			414S	31.73	0.12	0.2
			450S	30.61	0.10	0.3
C21orf45	Horn	Female	761S	27.19	0.16	1.5
			763S	29.80	0.06	0.4
			781S	28.41	0.29	0.9
		Male	780S	29.21	0.03	0.4
			786S	27.18	0.14	1.6
			787S	28.25	0.18	0.7
	Poll	Female	416S	28.77	0.32	0.5
			433S	26.35	0.38	2.7
			778S	28.24	0.18	1.0
		Male	729S	28.85	0.08	0.7
			751S	28.01	0.01	1.0
			777S	27.34	0.12	1.4
	Scur	Female	449S	28.15	0.13	0.9
			449S	28.15	0.13	0.9
			449S	28.15	0.13	0.9
		Male	358S	29.52	0.15	0.6
			414S	28.69	0.22	0.7
			450S	28.61	0.49	0.6

Table A.14. Continued

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
C21orf59	Horn	Female	761S	28.83	0.23	0.6
			763S	29.58	0.22	0.8
			781S	28.53	0.08	0.9
		Male	780S	28.98	0.12	0.7
			786S	28.82	0.03	0.7
			787S	28.23	0.06	0.9
	Poll	Female	416S	27.29	0.03	1.0
			433S	28.34	0.15	1.1
			778S	27.99	0.02	1.0
		Male	729S	27.94	0.04	0.9
			751S	27.56	0.04	1.0
			777S	27.71	0.03	0.9
	Scur	Female	438S	30.21	0.26	1.3
			449S	30.75	0.10	0.4
			739S	28.93	0.02	0.5
		Male	358S	30.56	0.11	0.7
			414S	29.08	0.01	0.8
			450S	28.48	0.08	0.5
C21orf62	Horn	Female	761S	25.03	0.05	1.6
			763S	25.48	0.14	1.2
			781S	25.66	0.07	1.0
		Male	780S	26.28	0.02	0.9
			786S	24.69	0.17	2.2
			787S	25.44	0.49	0.5
	Poll	Female	416S	26.48	0.08	0.6
			433S	26.78	0.27	0.4
			778S	24.95	0.11	2.3
		Male	729S	27.94	0.61	0.3
			751S	25.82	0.10	1.0
			777S	24.70	0.01	1.5
	Scur	Female	438S	26.11	0.14	1.1
			449S	27.90	0.34	0.3
			739S	26.94	0.30	0.8
		Male	358S	28.32	0.73	0.4
			414S	26.23	0.07	1.0
			450S	26.59	0.21	0.7

Table A.14. Continued

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
C21orf66	Horn	Female	761S	28.39	0.06	1.1
			763S	29.39	0.09	1.1
			781S	28.50	0.04	1.2
		Male	780S	29.09	0.10	0.8
			786S	28.43	0.02	1.1
			787S	28.28	0.04	1.1
	Poll	Female	416S	27.12	0.04	1.3
			433S	28.77	0.04	1.0
			778S	28.66	0.03	0.8
		Male	729S	28.08	0.02	1.1
			751S	27.81	0.05	1.0
			777S	28.41	0.00	0.8
	Scur	Female	438S	31.72	0.10	0.7
			449S	30.13	0.04	0.9
			739S	28.85	0.12	0.8
		Male	358S	30.91	0.06	0.7
			414S	28.91	0.09	1.1
			450S	28.37	0.11	0.8
COL18A1	Horn	Female	761S	27.48	0.05	10.8
			763S	28.69	0.15	4.3
			781S	29.43	0.05	4.7
		Male	780S	29.83	0.03	2.8
			786S	29.94	0.00	2.1
			787S	28.76	0.13	3.6
	Poll	Female	416S	30.04	0.14	1.3
			433S	27.59	0.03	10.3
			778S	26.53	0.05	12.4
		Male	729S	29.58	0.03	2.9
			751S	30.60	0.23	1.0
			777S	29.74	0.05	2.8
	Scur	Female	438S	28.12	0.06	17.6
			449S	32.39	0.24	1.0
			739S	30.62	0.09	3.2
		Male	358S	28.47	0.07	24.3
			414S	30.15	0.15	5.3
			450S	31.02	0.15	1.3

Table A.14. Continued

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
FOXL2	Horn	Female	761S	28.83	0.12	2.5
			763S	29.28	0.04	2.0
			781S	30.74	0.35	0.7
		Male	780S	30.38	0.08	1.2
			786S	28.66	0.19	3.1
			787S	28.53	0.22	2.4
	Poll	Female	416S	30.31	0.12	1.0
			433S	29.94	0.19	0.9
			778S	29.23	0.36	2.3
		Male	729S	31.77	0.24	0.5
			751S	30.30	0.25	1.0
			777S	28.93	0.16	2.9
	Scur	Female	438S	30.00	0.11	1.7
			449S	31.38	0.22	0.6
			739S	30.42	0.10	1.2
		Male	358S	30.91	0.04	1.3
			414S	30.54	0.02	1.1
			450S	31.92	0.58	0.4
IL10RB	Horn	Female	761S	28.93	0.04	0.6
			763S	29.54	0.36	0.7
			781S	28.14	0.12	1.1
		Male	780S	28.97	0.16	0.6
			786S	28.65	0.19	0.7
			787S	28.56	0.14	0.6
	Poll	Female	416S	27.12	0.09	1.0
			433S	27.98	0.10	1.3
			778S	27.72	0.07	1.1
		Male	729S	27.83	0.16	1.0
			751S	27.46	0.02	1.0
			777S	27.32	0.08	1.2
	Scur	Female	438S	31.76	2.14	0.4
			449S	29.94	0.22	0.7
			739S	28.32	0.10	0.7
		Male	358S	29.49	0.06	1.3
			414S	28.31	0.04	1.2
			450S	27.63	0.05	0.9

Table A.14. Continued

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
PRKCA	Horn	Female	761S	28.93	0.07	0.4
			763S	28.45	0.01	0.5
			781S	29.12	0.08	0.6
		Male	780S	28.96	0.01	0.5
			786S	28.71	0.17	0.5
			787S	27.78	0.05	0.6
	Poll	Female	416S	27.03	0.15	0.9
			433S	28.69	0.14	0.5
			778S	27.55	0.09	0.6
		Male	729S	27.56	0.03	1.0
			751S	27.22	0.06	1.0
			777S	28.30	0.08	0.7
	Scur	Female	438S	29.78	0.13	0.6
			449S	29.50	0.08	0.7
			739S	29.51	0.07	0.6
		Male	358S	30.70	0.18	0.5
			414S	30.27	0.09	0.5
			450S	28.52	0.05	0.7
PTHLH	Horn	Female	761S	31.51	0.22	0.5
			763S	31.65	0.33	0.4
			781S	30.99	0.14	0.6
		Male	780S	31.80	0.43	0.6
			786S	31.30	0.17	0.4
			787S	32.62	0.01	1.1
	Poll	Female	416S	29.55	0.01	1.2
			433S	30.11	0.32	1.1
			778S	31.37	0.19	0.8
		Male	729S	30.40	0.30	1.1
			751S	29.53	0.22	1.0
			777S	30.39	0.14	0.7
	Scur	Female	438S	31.27	0.15	1.4
			449S	31.45	0.14	0.8
			739S	30.77	0.34	0.9
		Male	358S	32.86	0.12	1.3
			414S	30.62	0.46	1.0
			450S	31.06	0.45	0.6

Table A.14. Continued

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
RUNX1	Horn	Female	761S	27.81	0.07	1.3
			763S	28.18	0.21	0.9
			781S	28.39	0.08	1.5
		Male	780S	28.57	0.19	0.9
			786S	27.66	0.04	1.5
			787S	27.79	0.09	0.9
	Poll	Female	416S	27.46	0.02	1.0
			433S	27.88	0.05	1.2
			778S	27.30	0.10	1.0
		Male	729S	28.41	0.06	0.8
			751S	27.77	0.13	1.0
			777S	27.94	0.04	1.4
	Scur	Female	438S	28.52	0.13	1.9
			449S	29.40	0.09	1.1
			739S	29.17	0.05	1.1
		Male	358S	30.33	0.11	1.0
			414S	29.76	0.10	1.0
			450S	28.32	0.03	1.2
RUNX2	Horn	Female	761S	25.97	0.13	3.6
			763S	25.31	0.10	3.6
			781S	27.38	0.08	1.1
		Male	780S	27.06	0.13	2.5
			786S	25.86	0.08	2.0
			787S	28.69	0.08	2.7
	Poll	Female	416S	26.63	0.09	1.2
			433S	27.53	0.28	1.0
			778S	26.83	0.01	2.2
		Male	729S	27.43	0.09	1.2
			751S	26.71	0.08	1.0
			777S	24.37	0.04	6.4
	Scur	Female	438S	27.44	0.73	3.1
			449S	28.17	0.14	1.3
			739S	26.95	0.05	1.3
		Male	358S	29.49	0.16	2.1
			414S	27.33	0.23	1.5
			450S	27.56	0.08	0.8

Table A.14. Continued

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
SFRS15	Horn	Female	761S	27.67	0.08	1.1
			763S	29.03	0.06	0.9
			781S	27.81	0.08	1.2
		Male	780S	28.60	0.05	0.7
			786S	27.95	0.02	1.0
			787S	27.65	0.11	1.0
	Poll	Female	416S	26.86	0.03	1.0
			433S	28.54	0.03	0.7
			778S	28.06	0.05	0.8
		Male	729S	27.24	0.03	1.2
			751S	27.23	0.06	1.0
			777S	27.47	0.06	0.9
	Scur	Female	438S	30.61	0.11	0.8
			449S	29.38	0.13	0.9
			739S	28.18	0.03	0.7
		Male	358S	30.06	0.06	0.7
			414S	28.38	0.06	1.0
			450S	27.83	0.05	0.7
SOX9	Horn	Female	761S	28.19	0.05	0.4
			763S	28.79	0.04	0.5
			781S	26.78	0.03	1.1
		Male	780S	27.32	0.04	0.8
			786S	27.63	0.07	0.6
			787S	28.57	0.02	0.3
	Poll	Female	416S	26.42	0.03	0.6
			433S	26.59	0.02	1.3
			778S	26.95	0.04	0.8
		Male	729S	26.95	0.02	0.7
			751S	26.03	0.03	1.0
			777S	26.71	0.05	0.8
	Scur	Female	438S	28.95	0.03	1.3
			449S	29.00	0.11	0.6
			739S	28.37	0.03	0.3
		Male	358S	29.17	0.05	0.7
			414S	28.88	0.06	0.3
			450S	27.38	0.01	0.5

Table A.14. Continued

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
STAT1	Horn	Female	761S	28.35	0.15	1.0
			763S	29.20	0.17	0.5
			781S	28.48	0.03	0.8
		Male	780S	30.20	0.23	0.5
			786S	27.52	0.10	1.2
			787S	30.83	0.11	0.9
	Poll	Female	416S	27.30	0.04	1.4
			433S	29.47	1.23	0.4
			778S	28.98	0.18	1.0
		Male	729S	28.36	0.06	1.1
			751S	27.47	0.08	1.0
			777S	27.92	0.10	0.9
	Scur	Female	438S	29.31	0.07	1.3
			449S	30.05	0.19	0.5
			739S	28.68	0.07	0.9
		Male	358S	32.04	0.09	0.5
			414S	29.26	0.11	0.6
			450S	28.59	0.10	0.8
SYNJ1	Horn	Female	761S	31.33	0.26	0.4
			763S	30.39	0.25	0.6
			781S	31.45	0.11	0.6
		Male	780S	31.10	0.18	0.6
			786S	30.61	0.09	0.7
			787S	30.20	0.15	0.6
	Poll	Female	416S	29.47	0.19	1.0
			433S	30.62	0.14	0.6
			778S	29.90	0.08	0.6
		Male	729S	29.89	0.07	1.1
			751S	29.56	0.29	1.0
			777S	30.67	0.06	0.7
	Scur	Female	438S	31.34	0.00	0.9
			449S	32.27	0.04	0.5
			739S	32.16	0.03	0.5
		Male	358S	33.56	0.00	0.3
			414S	32.65	0.16	0.5
			450S	31.17	0.13	0.6

Table A.14. Continued

Detector	Type[1,2]	Sex[3]	Sample	Ct Avg[4]	Ct SD[5]	RQ[6]
TWIST1	Horn	Female	761S	29.00	0.08	1.1
			763S	29.06	0.03	1.0
			781S	30.83	0.07	0.5
		Male	780S	30.63	0.15	0.4
			786S	28.84	0.07	1.3
			787S	28.31	0.12	1.2
	Poll	Female	416S	28.36	0.19	1.0
			433S	29.12	0.09	1.0
			778S	28.79	0.02	0.7
		Male	729S	29.06	0.06	1.0
			751S	28.73	0.11	1.0
			777S	28.91	0.08	1.4
	Scur	Female	438S	30.42	0.00	1.0
			449S	31.77	0.08	0.4
			739S	31.17	0.15	0.5
		Male	358S	32.75	0.06	0.4
			414S	31.21	0.09	0.7
			450S	30.46	0.10	0.5
TWIST2	Horn	Female	761S	30.32	0.05	0.6
			763S	30.64	0.12	0.5
			781S	30.25	0.24	1.1
		Male	780S	31.37	0.06	0.4
			786S	30.02	0.05	0.8
			787S	28.96	0.11	1.1
	Poll	Female	416S	29.06	0.07	0.9
			433S	29.31	0.10	1.2
			778S	28.89	0.06	1.0
		Male	729S	29.74	0.06	0.9
			751S	29.24	0.04	1.0
			777S	29.86	0.05	1.0
	Scur	Female	438S	30.69	0.06	1.2
			449S	32.24	0.06	0.4
			739S	31.72	0.33	0.5
		Male	358S	32.09	0.12	0.8
			414S	32.36	0.06	0.5
			450S	30.25	0.09	0.9

[1]Based on breed type and observation at time of sample collection
[2]Poll = skin sampled from the same location as horn or scurs were collected
[3]Males were castrated at 7 to 30 d old; 414S and 787S were left intact.
[4]Average cycle threshold value
[5]Standard deviation of cycle threshold
[6]Relative quantity

Table A.15. Gene specific primers used to construct probes for in situ hybridization

Primer	Forward	Reverse	Size
C21ORF59_IS[1]	GGCTCAACGTCATCAAAGAA	GGCAAACTTCATCTGGGTCT	343
IL10RB_IS	ATTAGGAATGGTTCCACCTC	CATGTAAAGAATTAGCAAGTGC	335
RUNX2_IS	TGAGCCAGATGACTCCTCCA	GGACATACCGAGGGACATGC	322
TWIST1_IS	TCCAGAGAAGGAGAAAATGG	GTCCATAATGATGCCTTTCC	360
GCF2.4[2]	GATGTCGATGTCGCACTGTT	CTGCATTCACCACTGAAGGA	166
TIAM1_EX	CGCAGAAAGTCAAAGTGTCG	CCCAAGATCTCTTCGTTGCT	594
SOX9[2]	TGCTCAAGGGCTACGACTG	CGAGATGTGCGTCTGTTCC	358
SFRS15[2]	AGTTCGTGTATTGAACCTTTG	GGTACAGTTGGTACAGAGTTTG	201
SYNJ1[2]	AGTTGCAATACGAATGCTGT	TGCTGTCTGATAAGCTCTTTC	234

[1]Reverse primer previously used for qualitative RT-PCR experiments
[2]Forward and reverse primers previously used for qualitative RT-PCR experiments

APPENDIX B

Breed consensus sequence used to assign SNP positions in the *C21orf66* sequencing project. Exons within the primary transcript of *C21orf66* are in bold and italicized, while exons within the putative alternative transcript are underlined with the start codon boxed.

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1       TCTAGTGACACAGTTAATGTGGACTACAGTCCTGGTCTCGGAGACCAGGAAAATATGAAC
61      AGTCGTATAAACAGAGCGATAAGAGGGAGGGGAGCGTGTTTGTCTAGTTAGACATTGAGG
121     TTTACCATTAACTGTTTCATCATCGGTATGCATGCTGCGTTAACAGACAGCACGTGGGTA
181     GCTGATTCCAACAAGAAACCGCTTGTCTCCTGAATTCCAGGAAGTCAGATATTGAGGGGG
241     AGGAGGGAATGTGCGTGCTTAAGTGGATAAGACAGACAGTACCCTCCAATCACTCCAATT
301     CTCCATCCACAGACAGGATGCACTGGGTCTCCTCCCCCCGCATCTACTCTTTGCTTGCTT
361     ATTGGCGTGTTCATCTCACCACCAACAGACACTACCGGGAGTTTTCTAGCTCACTTTTTC
421     AGATTTCAGGGGCCTGGTTGCTGGCCTCCCCCCACCCTCACCCCAGGCTCTTAGAAAACA
481     GCATGCCCTGGACTTGGGCTCCTAGCATCGCCTGCCTTTGCTGAAACTGCAAGTGAGAAA
541     GTTTACGACATGTTATTTCGGGGGTTCCTTTTTTTCCCAGAAGCTGCTCATTTCTAAGGA
601     GAGGCTGTGATAACAAAACTGCTTATTTTCTTTCAGGTGAGTTTCAATGAATCGGCTCAT
661     GAAGGGGAACCTGGCTGGCCCGCGCTGAGACGAGAATGGCACCTCCTTCTGGGCACAGCT
721     TCTTTCTGATCGGTGCGCTGGGCGTCTTTGCACTCAACTGCTTCACCAGAGCTCAGAGGA
781     ACGGCACGCTCATTTTCACCAAGGAAAACACCATTCGGAAGTGCAGCTGCTCAGCAGACA
841     TCCGCGACTGTGACTACAGTTTGGCCAACCTGATGTGCAGCTGTAAAACCATGCTGCCTC
901     TTGCCGTTGAGCAAACGAGCTACAGTGACCGTCTGACCATCTGGTTCACAGACACGTCTG
961     CGCTGGGGCTCCTGCTGAACTTCACGCTGGTCCGGGACCTGAAGCTTTCCCTGTGCAGTA
1021    CCAACACTCTCCCCACTGAGTACCTGGCTATTTGCGGTCTGAAGAGGCTTCGGGTCAGCA
1081    CGGAGGCCAAGCATCCCTCCCCTGAACAGAGTTTGCTCATCCACGACGGTGGGGAGAGTG
1141    AACCCAGAGAGAAACCCACGTTACAGCGAGGCTGGCAAACCTGTATGTATCTCTCCTTCT
1201    TAGACATGGCACTCTTCAACAGGGAGTCTGCCTTAAAATCATACAGTGTTGCAAACTCTG
1261    CCAGCGTGGCCAACAACTTCCCCTACTTTTCCTACCTTAAAACCTTCCCAGTTCTAAACA
1321    ACAAAAGCTATGTGGTCACCTTCATTTACTAATATCGTAGCTGTGCCCATCCTCCGGGGA
1381    CTTTGGCATCTGCTGGATCATCTCAACAAGGGATCTGTGAACAAGGTCATGGGTATTCCT
1441    AGCCTCAACTTCACTTCTCCTCCAGCCTCCTTTCACACAGAGCCAGCCCTCTGCTCCACC
1501    CACACCTGGCCCTGGGAAAACAAAATAAAAATAATAAAAAAAACCTACTTGATTCAGTCT
1561    ATAAACATATGTTGGGGGCCAGCTAGAGGCTAGGTTTTTGCTACTAAGGGAGGGTAGGGA
1621    AATGTCAGATCAGTGCACCCCAAGGACTGCTTAGTGCAGCTCAGCAGAGGGGATAAACCC
1681    TAAAGAAAGGAGTCTTCCAGATTTCGCTGGTGATGCAGCGATTGACACTTGCCTTTCAAT
1741    GCAGGGGGTGCCGATTTGATCCTTGGTCAGGGAACTAAGATCCCACATGCCTCGTGGCCA
1801    AAAAACCAAAATATAAAACAGAAGCAATGTGTAACAAATTCAATAAAGACTTTCAAAAAC
1861    TGAAAGGGAACTGAAAGTGTTAGTCGCCCATTCTTGTCTCACTCTCTGCAACCAGTGGAC
1921    TGTAGCCCACTAGGCTCCTCTAGCCATGGACTTCTCTAGGCAAGAACACTGGAATATGTA
1981    GCCATTTCTTTCTCCAGGGGATCTTCTCGACCCAGGGATCAAACTTGGGTCTCCTGGCAG
2041    ATTCTTTACAATCTGCCATCTGAGCCACCAAGGAAGCCCTTAAAAAAAAGAAAAGCATCT
2101    TCCTTCAAAACATTGCCTTTTTTGTCCCCTCCTTCATGTTCTTACATAGGCTGGAATTCC
2161    CCTACATAGAGAATGACAGACTTCAGTGGTTTTGCGACAAGGAGCCCCTCAAAATGATTA
2221    GATCCTCTTAGACTGGTATTTTCCAAACATCTTGTAAAGCACATTCACACTGAGAAAGGG
2281    AAGTCTTGTGAGTGTGTTCAGTGACGGGGAGTGGCGGGTGGTGCCGGTGGGGAGGGTTGA
2341    AGCCTGGCGGATATGGTGGAAACTTAGGGCTGAATAAGAACATCAGGGTCCGAGAGGGAA
2401    GTCACAAAAGCCAAGTGCATTTGGGATTTTTGTTGCTTAGAAAGAGGCCTAAATCCGGGG
2461    AATGCTCTCTCCCTCTAGCCCTTCTCTCCCTCTCTTGCCTTGCCACCTCCTACCTTTGGC
2521    CCAGCTACACAGCTTCCCCACTGTTCCTCCCCTCCTCCTCCTCCTGCTCCAGAAATCAGA
2581    GCTTAGATTAATACCACTGGAACTGTGTTTGACTACATTAGGGGCTAGCCTGGGAATGTA
2641    ATAACCCCTTTATAACACTACTCGCATGGGTAAATACATCCAGATTACTTATAGATAATT
2701    AACAGAAGAATTTTTAGGGCTCAGTCCATTTGGAAGTTTAAGACTGGAAAGTGAAGACAC
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Appendix B. Continued

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2761    TCTGTATGATGGGGTTAACATAAAATGCTTTTATGTGTGTTCATACAAAGAACCAAGGCA
2821    ATATTTTTCATTAATTTTCCCTGGAGAATAGCTGCTTTACAAAGTTGTGTCAGCTTCTGC
2881    TCTCAGCAAAGTGAATCACCAAACGGATACACGTATCCTCCCTTTTTTGGATTTCCTCCC
2941    GTTTAAGTCACCAGCAAGCACAGAGGAGAGTTCGCAGTGCTGGACAGTGGGTTTTCATCA
3001    GTTATCTATTCTATAGATAGTATCCATTTTTAAAAATTAAACTGACTCATCATTGGACAA
3061    AGAAAATCTTCCTTCACAGAAACCCAACTCCACACTAAAATGGGATATAAAGAATTTGTG
3121    GCAATTATGGTACAAACACCTAAACCCAGAAGGCAGAAAGTTGGAACGTATTGAATGCAT
3181    GATACCGTAATGATAAGAAATAGTTCTACCATGAAATAACACGTATTTTTCTCTTAGCCT
3241    TAACACCAGATAGCTGAAATAACCTGAAATGTTTCCATTTGCTTAACTGGTGAGAGATTT
3301    GGCCATTCAGACTAATTAGCATGCATCTATAATATCAGTGCTTGAATTAGGCCAGAACTA
3361    AGACCAAGATACCAATTTTAATAGAAAGATGTGATGACCTTATTACTGACATTGTATGTG
3421    AGAGGTAAGATTAAAGTGGTAGAAAAAGATAAATAAGGAAACAAAAGAGAGAGAAAGAAT
3481    GGCAAGAAGGATATAGATGAAAAGATTAATATTAAATGTATTTACTGTCATTAGTTACTG
3541    AAAATGTAACAAATGGAATTTTAATAATTTTTATTTATAAAGTATAAAATTTACTGATAC
3601    TACAAATTATGAGTATCTTAATGTGTATATCTTCCATTTGGTGACTTTTAATAGCTTCAG
3661    AGCTCTTTCACATATGTTTTAAGTAATTCTTACAAGAAATACTTCTTGGGTCCCCTCTGG
3721    TGGCTCAGTGGTAAAGAATCTGCCTGTCAATGTAGGGGACACAGGTTTGATCCCTGATCC
3781    TGGAAGACCCCACACGCTGCAGAGCAACTAAGCCTGCGTGCACAGCTACTGAACCGGAGC
3841    TCTAGAGCCCAGGAGTCGCAACTGCTGAGCCCTGCAACAGTTGGGGCTGTTGCAACAACT
3901    GTTGCAACAACAAAGCAGTTGCAACAACGCACTGCAATTACTGAAGCCTCTGCTCCCCAA
3961    CTGGAGAAGCCACCGCAACGAGAAGCCCGAGCACCGCATGCGAGACAGCCCCCGCCCACC
4021    ACAACTGCACAAAGCAACAAAGACCCGATGCAACCCAAAACAATGCTTTAACAAGCGAAC
4081    AAGAAAAACGTAACTAGTATACAGTGGAACTATATGTAACCTTGCTCTGCATTTTCTGAG
4141    TCTCCTATAAATGTGTTATTATGTTTTCATAATTAAAAAAAAACAACTCATGTTCCAAAT
4201    ACAAAAAAAAAAATTCTGAACACAATGATAAATTCATCTTTGATAGGAAGAAGGTCACTT
4261    TTTTCAGTTTCCTGAGTGAAGCCAAGAGAAGTACAGATGAAAGTGGGTGTGTAGATGTGG
4321    TTCCCTCCAATGCTTCTCTATTGTTAGTAAAGGATTAGCTAGTTATTAGCTGGGGATAGA
4381    AGGAAGGATTTGAGAAGAGGTGTATCATAATCTCATGGTTTGGCAAAGCTGGTAGCTCAG
4441    AGGTTAAAGCGTCTGCCTGCAACGCAGGAGACCCGGGTTCAATCCCTGGGTCGGGAAGAT
4501    CTCCTGGAGAAGGAAATGGCAACCCACTCCAGTATTCTTGCCTGGAAAATCCCGTGGACG
4561    GAGGAACCTGGTAGGCTACAGTCCATGGGGTCGCAAAGAGTTGGACGACTGAGCAACTCC
4621    ACTTTCTTTTCTTTCTATGGGAAAGTAAACTTAACAAGGGAACATTAAAAATACGGGACA
4681    GATAAATTTGTTGAAATCCCAAAGGTACAGATGGCTCTCTCATGATCTCTTCATTGCCAT
4741    ATGTTCACATGTTTTAAAACTTTGCCATTATTTCTCTGGAGTGATGGGAACAGGAAACGA
4801    TAAAAACACATGATTATTCCCATGTCATTAACTCAAAATCTATTTACCTCTTCCAAAACT
4861    TTCCCACTTCTTTGCTTATTGGTTTGTTAAGAGCCGTTGTTGTTGTTCTGTCGCTACGTC
4921    GTGTCTGACTCTTTGTGACCCCATGGATTGCAGCACTCCAGGCTTTCCTGTCCTTCACCA
4981    TCACCTGGAGCCTGCTCAAACTTGTGTCCATTGAGTCGGTGATGCCATCCAGCCATCTCA
5041    TCCTTCGTCGTCCCCTTCTCCTCCTGCTTTCAATCTTTCCCAGCATCAGGGTCTTTTCCA
5101    ATGAGTCAGCTCTTCGCATCAGGTGGCCAAAATATTAGAGCTTTACTGTCCAATATTCCA
5161    GTGGATATTCAGGGTTGATTTCTTTTGGGATTGACTGGTTTGATCTCCTTGCTGTCCAAG
5221    GGACCCTCAGGAGTTTTCTCCATTACCACAATTTGAAAGCATTATATTCTTCTTAAATCA
5281    GTTGTGAAATTTGTATTTTGCCAAAATATACATTTTCTGTAGAATTTCCAATGTATTGTC
5341    ACTGAGTACCATACAACATTTTCTCACAATTAATTTGGCTCTTCGTCATGTCTACTTATA
5401    GTTAAAGTTTTGGGGGATATGTATGTTCCTTAATTAAGTTTGAGTTTTTCTTGGTAATCC
5461    CTATGTTTGGGGTTTTATTTTATTTTCTATTTATTTTTTATTAGTAATCATTTTATCTTT
5521    GACTATTTCTACTATACACATTCTTTGAATTGTTTTGCTGTTTTTTTTTCTTTGTTTTTC
5581    TGAGGCTGAATATTTCTCTTTTCACTTGTGTAATTATAAACTTAGTTTATTTTTTTCCTT
5641    TTTTAAAATATGAATTTATTTATTTTAATTGGAGGGTAATTACTTTACAATATTGTAGTG
5701    GTTTTGCCATACATTGACATGAATCAGCCATCAGTGTACATATGTTCCCCATCCTGAACT
5761    CCCCTCCCACCTCCCTCCCCAACCCATCCCTCTGGGTCATCCCGGTGCACCAGCCCTGAG
5821    CGCCCTGTCTCATGCATCGAACCTGGACTGGCAATCCATTTCACATATGATAATATACAT
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Appendix B. Continued

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5881    GTTTCAGTGCTATTCTCCCAAATCATCCCATCCTTGCCCTCTCCCACAGAGTCCAAAAGA
5941    CTGTTCTATACATCTGTGTCTCTTTTGCTATCTCACATATAGGGTTATCGTTACCATCTT
6001    TCTAAATTCCATATATATGTGTTAGTATACTGTATTGGTGTTTTTCTTTCTGGCTTACTT
6061    CACTTTGTATAATAGGCTCCAGTTTCATCCACCTCATTAGAACTGATTCAAATGTATTCT
6121    TTTTAATGGCTGAGTAATACTCCATTGTGTATATGTACCACAGCTTTCTTATCCATTCAT
6181    CTGCCGATGGACATCTAGGTTGCTTCCATGTCCTGGCTATTATAAACAGTGCTGTGATGA
6241    ACATTGGGGTACACGTGTCTCTTTCAATTCTGGTTTCCTTGGTGTATATGTCCAGCAGTT
6301    GGATTGCTGGGTCATATGGCAGTTCTATTTCCAGTTTTTTAAGGAATCTCCACACTGTTC
6361    TCCATAGTGGCTGTACTAGTTTGCATTCCCACCAACAGTGTAAAAGGGTTCCCTTTTCTC
6421    CACACCCTCTCCAGCATTTATTGCTTGTAGACTTTTGGATAGCAGCCATTCTGACTGGCG
6481    TGAAATGGTACCTCACTGTGGTTTTGATTTGCATTTCTCTGATAATGAGTGAGGTTGAGC
6541    ATCTTTTCATGTGTTTGTTAGCCATCTGTATGTCTTCTTTGGAGAAATGTCTGGTTAGTT
6601    CTTTGGCCCATTTTTTTGATTGGGTTGTTTATTTTTCTGGAATTGAGCTGCGGGATTTGC
6661    TTGTATATTTTTGAGATTAATTCTTTGTCAGTTGCTTCGTTTGCTATTATTTTCTCCCAT
6721    TCTGAAGGCTGTCTTTTCACCTTGCTTATAGTTTCCTTTGTTGTTCAAAAGCTTTTACGT
6781    TTAATTAGGTCCCATTTGTTTATTTTTGCTTTTATTTCCAATACTCTGGGCGGTGGGTCA
6841    TAGAGGATCCTGCTGTGATTTATGTCGGAGAATGTTTTGCCTATGTTTTCCTCTAGGAGT
6901    TTTATAGTTTCTGGTCTTACGTTTAGATCTTAATCCATTTTTAGTTTATTTTTGTGTATG
6961    GTGTTAGAAAGTGTTCTAGTTTCATTCTTTTACAAGTGGTTGACCAGTTTTTCCAGCACC
7021    ACGTGTTAAAGAGATTGTCTTTTCTCCACTGTATATTCTTGCCTCCTTTGTCAAAGATAA
7081    GGTGTCCATAGGTGCGTGGATTTATCTCTGGGCTAAAGTTATACATTATTCTGTGAGTCC
7141    CCCTTTAGGTATGTACCATATAGGGAAATGACACTTTTATATGTCATTATAAGTGTCATA
7201    AAAATGACACTTTTATTAATTTCTAGAGATCTTAGATAATGTATGTCTTATTTCCCCTTT
7261    GATTTAGAGATTTAGATGACTGTTCATTAATTCCCAAATAGCTAAGACTTTTTTTTTTGT
7321    ATTGGAGGGGTCATTCAGCTTCCTGGATTTGGCTCATCATTTAACTTTTTTCTCTGGTTG
7381    GAGAACGTTCCCTGTAAAAATCTCTGTTTTGAAGAATTTGTTCAGTTATTTGTTTACCCA
7441    GGTTAATGATTGATATTTATAAATGTTTCACAGATGCCATGGGTAATTAAATAGTCTCTG
7501    TTTGGAGAGAACATAATTTTATTAATCTATATATTTGAGCTCACTGATTATATTATTAAA
7561    ATCCTATATAAACTTATGCATTATTTGACTACTTCATCTGCCTGATTCAGACAGAGACCC
7621    ACATACAAATAATAATAAAAAATAATGTATTTAGTGTCCATTTGATGTCTTCAGTTAATT
7681    TAAGAGGAAATCTGGAAAGTTAACTAACCAGGGACAGATACAGAGAAGGTCACAGTCTGG
7741    CTCAGCTATGGCTAGGGTTTTTCTGAATGAGTACAACGAACCATTCAATCCTAAAGGAAA
7801    TCAACTCTGAATATTCATTGGGAGTACTGATGCTGAAGCTAAAACTCCAATACTTTGGCT
7861    ACCCGATGGAAAGAGTCAACTCATTGGAAAAGACCCTGATGCTGGGAAAGATTGAAGGCA
7921    GGAAGAGAAGGGATGAGAGGATGAGATCAGAGGATGAGATGGTTGGACTGAATCACCAGC
7981    TCAATGAGTTTGAGCAAACTCCAGGAGATGATAAAGGACAAGGAATCCTGGCCTGCTGCA
8041    GTCCATGCGGTGGCAAAGAGTTGGACAGGACTAAACAACAACAACAAAGCAGGAGAGGGG
8101    AAGGAAGCTGAGCTTGTCGGAAGGGAGTGACTATAATCTTGTCCCCATGAAATCCAATCT
8161    GGATGAGGCAGGAAACAAAGGCAGGAGGAAGAGAATGGGCAGTGAAAGGTGATGGGTTCT
8221    GGACTGGAGGTGGAATAGACAAACTAGAAGATGGAAGTGAAGTTTTACCAAAGTGAAAGA
8281    GAAGTCGCTCAGTCGTGTCCGACTCTTTGCGACCCCATGGACTGTAGTCTACCAGGCTCC
8341    TCCCTCCATGGGATTCTCCAGGCAAGAGTACTGGAGTGGGTTGCCATTTCCTTCTTCATT
8401    GAGAAGTTGGAGTGAGTTGGAAGTTTTACCAAGGAAGATGCCAAGTAACTCCCTAGTAAC
8461    TTGGAGAAGATGCAAGTTGCAAACTCCAAGATGCAGTCCTTATGATAAGATTAGAGTGAG
8521    GAGGTTAGAGAACTAACTCCAAGTAGAAGTTGCACTGGAATGTTGAAAACATGAAGAATG
8581    AGAGAGTGAATGTTCATAAAATAATTCTGGAAAGAAATGCTCCAAAAATTAAGTGTTAAG
8641    ATTAATAACAATTATTTCCTTCTTTGTATTGTTCTGCATTTTCAAAATTTCTAAAATGTA
8701    GTAATGCAGGTAGCACTTTTATAATTAGATAAGGGACTTCCCTAGTGGCTCAGATGGTAA
8761    ACAATTCGCCTGCAATGCAGGATACCTGGGTTCATTCCTTGGGTTGGGAAGATCCCCTGG
8821    AGAAGGGAATGGCTACCCACTCCAGTATTCTTGCCTGGAGAATCCCATGGACAGATGAGC
8881    CTGGTGGGCTACAGTCCACGGGGAAGCAAAGCGTCGGACAGGACTAAGCAACTAATACTT
8941    TCAAAGGTTATTTTTAAAAGTCCACTTCATTTTTTCAAAAATTTGGAGCTTGGGGACTTA
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Appendix B. Continued

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9001    TCTGGTGGTCCAGTGATTGAGAATCTGCCTGCCAGTGCAGGAGAGACAGGTCTGATCCCT
9061    GGTCTAGGAAGATTCTGCTTGCCAGAACACTCTAGAGCCCGTGATCCACAATACGAGAAG
9121    CCACCAGGATGAGACGCTCATGAACCGCAGCTAGAGTGTAGCCCCCTCTCACTGCAGTAG
9181    AGAAAGTCCACAAGCAGCAGTGAAGACTAGTGCAACCAAAAATAAACAAGCAAACAGGAT
9241    ATTTTAAAAGATCTGAAAAATTCAAAGCTTGATTTGATGCCTCACTCTGTAATCATACCA
9301    TACTGTTACTCTGTATTTCTTGCGCAATTTTGCTGCCCTGGAGTAAAATTAAATAGCACA
9361    GAAAAAAGACACATTCCTTCATCTTTCCTCCCAAAAGAGATACATGTAACTCTGTTGTCT
9421    TTGATAAGGTATCTGCTTTTGACTTTTTCCCCAAGAAGGAAAGACAACAATGTCTGAACG
9481    ATTTTGAGCTAATAGAGGTGAATTCTCTTAGAAAGGATGCATAGCTATTTTTGCTTCTGT
9541    ATTGGTTGTATAGTCCTTAGATGTATTTATAAAGAGTTTATTGTGAATGTAATATAAATA
9601    TCACATGGTACTATGATTATGAATTGTACTTGTTGACATACTAACACCGGAAGATATAAA
9661    ACACTAAATCAGACAAAATGATTTGTTATTATTCAGTCACTAAGTTGTGGCTGACTCTCT
9721    GTGAGCCCGTGGACTCAGCACGCCAGGGTTCCCTGTCCTTCACTATCTCCCGGAGTTTGT
9781    TCAAACTCATGTCCATTGTGTCGATGATGCGATCCAACCACCTCATCCTCTGTTCTCCCT
9841    TCTCTTTTTGCCCTCAAATTTTCCCAGCACCAGGTTCTTTTCCAATAAGTCAGCTTCTTG
9901    CATCAGGTGGCCAAAGTATTGGAGAAGGGAATGGCAAACTACTTCAGTATTCTTGCCTTG
9961    AGAACCCCATGAACAGTATGAAAAGGCATGAATAACACTGGAGTGGGTTGCCATTTCCTT
10021   CTCCAGAGGATCTTCCCAACCCAGAGATGGAACCTGCCTCTCCTGCATTGCCAGCGGATT
10081   CTTCACCACTGAGCCACCTGGGAAGGACTGTGAAGCTAGCAGTGAAAAAAACAAACAAAC
10141   AAACAAACAAAATCTCCTCCCCCAGGAACCTTACATTTTACATTAAGTTAATGCAAAACT
10201   GTCTTCGCTAAATCAAGGACAAGAAATTTAATGAAATAATTAAATGTTTTCTCCAGCTTT
10261   TTGCCCCCCAGAATAGAATTCTTGAGTTATTTAGGAAGAAAGGAAAAATTTGTAGACCAG
10321   GAATTCCTAGACTATTAGATTTTGCATGCCACATAAAATTTTTAGATATAACAAACAATT
10381   TGGGGGGACTTATAGCTTTCACTACTTTTCAGTTGGTCAAATAAGGGTATGAAAACAAAC
10441   AAAATATACCATCATCATTATTTCATTTAAGGAAAGAGATTTTAGTATCTTAGTATGGAA
10501   GGAGGAAGATGACCTCAGAACAAAGCACCATTTTCTCCCCCCTGATAAGAGACATCTGGC
10561   TACTATACATGAGCAGACTGTTCGTGTGCTTCTCATTTGGCAACTGACTAGGGAAAACTT
10621   CACGGGCTCACAGAGCTACATTTGGTAATCATTGTCTCAGGTTACAGAGTCTAATACTGA
10681   GTTGGCAAACAAAACAAACAAAAGTGGTTTGCCTATTTTGTCTGAAATCATGGGTCAGTT
10741   ATTCCATGCAGAGTCACAATTTAGTAGAAAAAAAAACTAATTGGGATTATTGCTTGGAAA
10801   GGTAACTTTAAAAAGCACTGGGTATGACACATATGCACAGTGGAATATTACTCAGCTATA
10861   AAAAGGAATACGTTTGAGTCCAAACGAGGTGGATGAACCTAGAGCCTATTACACAGAGGG
10921   AAGTAAGTCAGAAAGAGAAAGACAAATATCATGTATTAATGCATATACATGGAATCTAGA
10981   AAGATAGTACCTACGATCCTACTGGCAGGGTACCAAAGGAGACACCGATATTTTGTGACA
11041   CAGTGGGGGAAGGAGAGGGTTGGATGATATGAGAAAATAGCATGGAAACATACATTACCA
11101   TATGTAAAAAAGATAGCCAGTGGGCATTCGAGGTGTGACACAGGGAACCCAAAGCTGGTG
11161   CTGTGTGACAACCTAGAGGAGAGGGATGGGAAGGGGGGGTTTCAAGAGGGAGGGGACATA
11221   GGTATATCTAATGTTGACTCATGTTGATGAATGGCAAAAACTGTCACAGTACTGTAATTA
11281   CACTCTAATTAAGAAAAAAAAAATCACTGGGGACCTCCCTGGCAGTCCAACTAAGACTTT
11341   GCCTTCTGATGAGGGGGTGTGGGTTCGATCCCTGGTCGAGGAGCTAAGATCCCACAGGGC
11401   TCACAGCTAAAAAATGAAGCGTAAAACAGAAGCAATATAAACAAAAAATTCAAGAAAGAC
11461   TCTCAAAATGGTCCACGTGATCAAAAAATACAACAAAATTTATTTTCTTAAAAAAGCACT
11521   GAACTGATTCAAACAATGATGGACGCAAGCATTCTACGAAAGCGGTGTTTGTCTAAAAAC
11581   ACTCCAACATTAAATAGTGATGCTATGCTGTAAACAGTCAGTTTAATACTCAGTGCACAG
11641   AGCAGTTTATAAAATAATTAGTTTCAGATCATATACTGGCCGACAGGCGATGAAAGCAGC
11701   TCTTCTGTTTCAGGAATTTAATTTCTACAAGGTCTGTAATCTCTAGAAGGGCCTTTGACT
11761   TGTGTTAGCAGCAAGATAACTTCTGAATCTTTAACTTTGCCCTTCAATTATCTGAATAGG
11821   TAGATTATAAAAGTTATAAATACATCGTGGCTTTTTATTTGTTAGCTTGATTTAAAACAA
11881   CCAACATTCTCCTCTCTATCCATATTTAATCATCACTTTCATACTGGAAGACCGTTTTGA
11941   AGTTTCAGGCCAAGATATTAACACAGTCTGCTCTGAACCTTATCTTTCTTACCTCTGTCC
12001   TAATCATGTTACTGCAAGTTTGTCTTTTGGCACTGGCACTGGTGTCTACAGGCTGACCAG
12061   GCACAATTTGAAGCTTTTGTGATGAGAACTGGAGTTCAATTTCACCAACGAAACACTCTG
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Appendix B. Continued

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12121   CTGGAATCAGAACTTAATGCTGAACAGCCAGAATATAAGAACTTAGAGTAATGCGAGTAG
12181   AATGGAAGCCAGATGTTATTAACTTTCTCTACTAACTGGAATAAAATGTTACAGAGGACA
12241   CTCTCAGCCTAGATCATCTTTCTAGCTTTCAAAAGTGAGTCACGGGTGGGCAGATTTCAA
12301   ATGCAATCAAAGAAAACCAGTGAAAAGAGCCAGTTGTGTATGAAATCTTGAGCTTGAAAT
12361   ATTTTGCTTCAGCCTGCAACTTAAATCTCAAGTGAAATTTTGACAGAATGGTGTAGGCAA
12421   ACAGACACTGTGGTAGGAGTACAAACTGGCATGGTTGGGAGTGGGCGAAATGAATGAATG
12481   GGGTTAAAACGTATATAAGTCATGGGAATGTAGTGATAAAATATAACTTGATTTGGTAAA
12541   AAATACTATATTACAGAAAAATACTATATTACATATTTGAAAGTTGCTAAGAGAATGGAT
12601   CTTAAAAGTGCTCATGGCAAGACAAAAACTCTGTAGCTACACGTGATGAAAGTTAACTAA
12661   ACTCTTGTGATCATTTTTCAATATATGTAAATATTATATCATTCTGTTGTACATGTGAAA
12721   CCAATATAATATTATATGTCAGGTGACTTCATTTGTATTTCCCTGGTGGCTTAGTGGTAA
12781   AGAATCTGCCTGTAATTAGGAGATGTGGGTTTGATCTTTGGATCAGGAAGGTCCCCTGGA
12841   GAAGGAAATGGCAACCCATTCCAGTATTCTTGCCTGAAATCCCATGGACAGAGGAGTTTG
12901   GCAGGCTATAGCCCATGGGGTTGCAAAAGAGTTGGACGTGACTTAGCAACTGAACAACAA
12961   TTACATAAAAAAAAAAAAATTTTTTTTTTTAAATTGCATGCTGCTATAGAGAGCGGCTTAG
13021   AATTGGCAAAATTACAAATGCACACCTGTGGATCTAGTAATTTCAATACCAATTCATCCT
13081   ACTCATGACCATTTTTCAAAGCTTTTCATCACAGTATTGATTATCATAGCAAGATTGGAA
13141   AAAACATTAATACCAATTCTTACGAAACTACTTAAATATTAATAAATTATGGTACATTTG
13201   AACAATGGATCACTATGCAGGTTGGTAGAAGGGACAACACTTTATGAACAGATATGAACT
13261   GATGTCCAATGTATGTTAGACTAAAAAAGTAAGTTACACATTGTATTAAAAAAAGGTAAT
13321   TCATAAAATACACATCTTCATTTTTATGTTCAAAGAATATCTCTTGTTGTTCCATCACTA
13381   AGATGTGTCCGACTGTTTGTGACTCCATGAACTGAAGCATGTCAAGCTTCCCTGTCCTTC
13441   ACTATCTCCGATAGTTTGCTCAAACTCATGGCCATTGAGTCAGTAATGCCATCCAACCAC
13501   CTCATCCTCTGCTGCCCCCTTCTCCCGTCCTCAATCTTTCCCAGCATCAGGGTTTTTTCC
13561   AATGAGTTGGCTCTTCAAATCAGGTGGACAAAGTACTGGAACTTCATCTTCAGCATCAGT
13621   CCTTCCAGTGAATATTCAGGGTTGGTTTCCTTTAGGAGTGACTGGTTTGGTCTCCTTGCA
13681   GACCAAGGGACTCTCAAGAAGATGAGTCTTCTCCAGCACTACAATTCAAAAGCATCAATT
13741   CTTCAACACTCAACCTTCTTTATGGTCCAACTCTCACATCTGTACACAACTGTTGGAAAA
13801   ACCATAGGTTTGACTATCCAGACCTTTGTTGACAAAGTGATGTCTCTGTTTGTAATATGC
13861   TGTCTAGGTTTGTCATAGCTTTTCTTCCAAGGAGCAAGTGTCTTAACTTCATGGCTGCCA
13921   TCAACATCTGCAGTGATTTTAGAGCCCAAGAAAATAAAATCTGTCACTGTTTTCACTTTT
13981   CCCCCATCTATTTGCCATGAAGTGTGGGACTAGATGCCATGGTCTTCGTTTTTTGAATAT
14041   TCAGTTTTAAGCCAGCTTTTTCACTCTCCTCTTTCACCTTCATCAAGAGGCTCTGTAGAT
14101   CCTCTTTGCTTTCTGCCATAAGGGTGGTGTCATCTGCGTATCTGAGGTTATTGATATATC
14161   TCCTGGCAATCTTGATTCCAACTTGTGAGTCATCCAGCCCAGAATTTTGTATGATGTACT
14221   CTGCATATAAGTTAAATAAGTAGGATGACAATTTACAGCCTTTTTGTACTCCTTTCCCAA
14281   TTTTGAACCAGTTCATTGTTCTATGTCTGGTTCCAACTGTTGCTTTTTGTCCTACATATA
14341   GGTTTCTCAGGAGACAGATGAAGTCGTCTGATATTCCCATCTCTTTAAGAATTTTCCACA
14401   GTTTGTTGTGATACACATAGTCAAAGGTTTTAGCGTAGTCAATGAAATGGAAGTAGATGT
14461   TTTTTCTTTTTTGGAACTCTCTTACTTTTTCTATGACCCAACAGATGTCGGCAATTTGAT
14521   CTCTGCCTTGTCTAAATTCAGCTTTTACATCCGCAAGTTCTTGGTTCACATACTGCTGAA
14581   GCCCAGCTTGATGGATTTTGAGCATTACTTTGCTAGAATGTGAAATGAGTGCAACCACAC
14641   GGTAATCTGAACATTCTTTGGCATTGCCTTTCTTTGGGATTGGAATAAAAGCTGACTTTT
14701   CCAGTCCTGTGGCCACTGCTGAGTTTTCCACATTTCCTGGTGTACTGAGTGCAGCACTTT
14761   CACAGCATCATCTTTTAGGATTTGAAATAGCTCAGCTGGAATTCCATAACCTCCACTACC
14821   TTTGTTCATAGTGATGCTTCCTAAGGCCCCCTTGACTTCACACTCCAGGATGTCTGGCTC
14881   TAGGCGTGTGACCACACCATCGTGTTTATCTGGGTCATTAAGACATTTTTGTACAGTTCT
14941   GTGTATTCTCTCCACCTCTTCTTAATCTCTTCTGTTTCTGTTAGGTCCTTGCCGTTTCTG
15001   TCATTTATTGAGCCCATTTTTGTAGGAAATGTTCCATTGGAATCTCCAGTTTTCTTGAAG
15061   AGATCTCTAGTCTTTCCCATTCTATTATTTTCCTCTATTTCTTTGCATTGTTCACTTAAG
15121   GCTATCTTATCTCTCCTTGCTATTCTCTGGAACTCTGCATTTAGTTGGGTATACTTTTCC
15181   TTTCTCCTTTGCCTTTCACTTCTTTTCTCCTGCTATTTGTAAGGCTTCTTTAGACAACCA
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Appendix B. Continued

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15241   CTTGCCTTTTGGCATTTTTCTTTGGGATGGTTTTGGTCACCACCTCCTGTATAATGTTAC
15301   AAACCTACATCCATAATTCTTTAGGCGTTCTATAAGATTTAATCCTCTGAATCTATTCAT
15361   TACCTTTACTGTATAATCATAAGGGATTTGATTTAGGTCAGACCCAGATAATCATGATGG
15421   TGTGATCTCACCTAGAGCCAGATATCCTGGAATGTGAAGTCAAGTGGGCCTTAGAAAGCA
15481   TCACTACGAACAAAGCTAGTGGAGATGATGGAATTCCAGTTGAGCTATTTCAAATCCTGA
15541   AAGATGATGCTGCAAAAGTGCTGCACTCAATATGCCAGCAAATTTGGAAAACTCAGCAGT
15601   GGCCACAGGACTGGAAAAGGTCACTTTTCATTCCAATCCCAAAGAAAGGCAATGCCAAAG
15661   AATGCTCAAACTACTGCACAATTGCACTCACCTCACATACTAGTTGGAGAAGGCAATGGC
15721   ACCCCATTCTAGTACTCTTGCCTGGAAAATCCCATGGATGGAGGAGCCTGGTAGGCTCCA
15781   GCCCATGGGGTGGCTAAGAGTCGGGCATGACTGAGCGACTTCACTTTCACTTTTCACTTT
15841   CATGCATTGGAGAAGGAAATGGCAACCCACTCCAGTGTTCTTGCCTGGAGAATCCCAGGG
15901   ACAGGGGAGCCTAGTGGGCTGCCCTCTATGGGGTCGCACAGAGTCAGACACGACTGAGGT
15961   GACTTAGCAGCAGCAGTAGCAGCACATGCTAGTAAAGTAATGCTCAAAATTCTCCAAGCC
16021   AGGCTTCAGCAATATGTGAACCGTGAACTTCCAGATGTTCAAGCTGGTTTAAGAAAAGGC
16081   AGAGGAACCGGAGACCAAACTGCCAACATCCGCTGGATCATCAAAAAAGCAAGAGAGTTC
16141   CAGAAAAAACATCTATTTCTGCTTTATTGACTATGCCAAAGCCTTTGACTGTATGGATTA
16201   CAATAAACTGTGGAAAATTCTGAAAGAGATGGGAGTACCAGACCACCTGACCTGCCTCTT
16261   GAGAAATTTGTATGCAGATCAGGAAGCAACAGTTAGAACTGGACATGGAACAACAGACTG
16321   GTTCCAAATAGGAAAAGGAGTTCATCAAGGCTGTATATTGTCACCCTGCTTATTTAACTT
16381   ATATGCAGAGTACATCATGAGAAACGCTGGGCTGGAAGAAGCACAAGCTGGAATCAAGAT
16441   TGCCGGGAGAAATATCAATAACCTCAGATATGTAGATGACACCACCCTTATGGCAGAAAG
16501   TGAAGAGGAATTAAAAAGCCTCTTGATGAAGGTGAAAGAGGAGAGTGAAAAAGTTGGCTT
16561   AAAACTCAACATTCAGAAAACTAAGATCATGGCATCTGGTCCCATCACTTCATGGGAAAT
16621   AGATGGGGAAACAGTGGAAACAGTGTCAGACTTTATTTTTTTGGGCTCCAAAATCACTGC
16681   AGGTGGTAACTGCAGCCATGAAATTAAAAGACGCTTACTCCTTGGAAGAAAAGTTATGAC
16741   CAACCTAGATAGCATATTGAAAATCAGAGACATTACTTTGCCAACAAAGGTCATCTAGTC
16801   AAGGCTATGGTTGTTCCAGTGGTCATGTATGGATGTGAGAGTTGGACTGTGAAGAAAGCT
16861   GAGCACCAAAGAATTGATGCTTTTGAACTGTGGTGTTGGTCTTGAGAGTTCCTTGGACTG
16921   CAAGGAGACCCAACCAGTCCATTCTGAAGGAGATCAGCCCTGGGATTTTTTTGGAAGGAA
16981   TGATGCTAAAGCTGAAACTCCAGTACTTTGGCCACCTCATGCAAAGAGTTGACTCATTGG
17041   AAAAGACTCTGATGCTGGGAGAGATTGGGGGCAGGAGGAGAAGGGGATGACAGAGGATGA
17101   GATGGATGGATGACATCACTGACTCGATGGACATGAGTCTGAGTGAACTCCGGGAGTTGG
17161   TGATGGACAGGGAGGCCTGGCGTGCTGCGATTCATGGGGTCACAAAGAGTCGGACACGAC
17221   TGAGTGACTGAACTGAACTGAATGGTCTAGTGGCTTTCCCTACTTTCTTCAGTTTAAGTC
17281   TGAATTTGCAATAAGGAGCTCATGATCTGAGCCATAGTAAGCTCCCAGTCTTGGTTTTGC
17341   AGATTGTATAGAGCTTCTCCATCTTTGGCTACAAAGAATATAATTAATCTGGTTTTGGTA
17401   CTCAGACGGTAAAAGCGTCTGCCTATAATGCAGGGGACCCGGGTTCGACCCCTGGGTCAG
17461   GAAGATCCCCTGGAGAAGGAAATGGCAACCCACTCCAGTACTCTTGCCTGGAAAATCCCA
17521   TGGATGGAGGAGCCTGGTAGGCTGCAGTCCATGGGGTCTCGAAGAGTCGGACAGGACTGA
17581   GCGACTTCACTTTCACTTTCTATTGAGCATCTGATGATGTCCATGTGCAGAGTTGTCGCT
17641   TGTGTTGGAAAAGCGTGTTTGCTATGATCAGTGTGTTCTCTTGGCCTTTGCCCTGCTCCA
17701   TTTTGTACTCCAAGGCCAAACTTGCCTGTTGCTCCAGGTATCTCTTGACTTCCTACTTTT
17761   GCATTCCAACCCCCTATGATGAAAAGGATCTCTTTTTGGTGTTAGTTCTAGAAGGTCTTG
17821   TAGGTCTTCATAGAAAGAATCAACTTCAGCTTCTTCAGCCTCAATGGTTGGGGTGCAGAC
17881   TTGGATTACTGTGATACTGAATGGTTTGCCTTGGAAACGAACTGAGATCATTTTGTTGTT
17941   TCTGAAATTGCATCCAAGTACTACATTTCAGACTCTTCTACTGACCATGAGGGCTACTCC
18001   ATTTCTTCTAAGGGATTTTTGCCCACAGTAATAAATATAATGGTGTGAATTAAATTCCTG
18061   TGATCCCACTAAAGACTGAGATAGATCCGCCTGTCAGCGTTGAAGAGCTCCTGCAGCGGT
18121   ATGGGTTGGCAGTAGCCTGCCTGGGGGACAGGGTCACCGGCAGCAGCAATCCTGGGAGGC
18181   ATGTATTGGCATAAGTCCTTTTTGAAGGTTGCCAGTAGTTCTACCATAGAGCCTGTAGAC
18241   TCTAGTATTGAGTTGCATCAGGGCATAACTAACATAGCATAGATCCAAATATCAGCAGAC
18301   AATTGGATTAAAGCTTTACTGAGCATGGCCCTAACCACCAGAGCAATACCCAGATTTTCC
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Appendix B. Continued

18361 CACAACCAGTCTCTCCCACCAGGAAGCAAGAACTATAATCCCACACCCTCCAGAAAACCA
18421 GGATCACACAAAGCTAACCAAAATCATCACACAGATAACAGTCTTGTTCTCAAGGATGCT
18481 ATCAGCCACCTGAGGGCCACCCAAGACTTGACAGCTCATGGTGGAGAGTTCTGAGAAAAC
18541 AGTCCACTGGAGAAGGGGATGGCAAACCACTTCAGTATTCCTGCCTCGAAAACCCCATGA
18601 ACAGTATGAAAAAACAAAAAAAAATATGACACTGAAGGATAAGCTCCCCAGGTCAGTAGG
18661 TGTCCAATAGGCTACTGTGGAAGAGCAGAGAAATAACTCCAAAAAGAATGAAGAGGCCGG
18721 GCCAAAGTGGAAATGTAGCTAAGTTGTGGATGTGTCTGGTGGTGAAAGTGAAGTCTGATA
18781 CTGTAAAGAACAATATTGCAGAGGAAGCTATAATGTTAGGTCCATGAATCAAGGTAAATT
18841 AGGTTTAGTCAAGCAGGAGACGGTAAGAGTCAACACCAACATTTTAGGAATCAATGAACT
18901 AAAATGGACGGGAATGGGTGCATTTAATTGAGAAGTCTTCTGGAGGATCACAAAAAGTTC
18961 AACATTGCCAGCGAACTGGAAAGTGCCTAGAAATGGATGTGAATACCTCTTGTAACCTTT
19021 TCAATTTTGAAACAAGAAAATTCACATATAGGCACATAGGCGAAGGAGAAGGCCCTACAT
19081 TCGGTGACACAGTGAGAAAGTAGGTGAACTCAACTTCAGAGTCAATGTGCCCTTGCCAGT
19141 TTAAAGGGTTACCTCTGTGACATGTCTGAAATGTGTCTCGCTACGTCGATTTTTTCCTAC
19201 ACTTGTTTCTGAAATCAGCCAAGTTGAGGAACGGCTAGCAAACCTGGCCTTCTTTTGAAA
19261 AAATCAAGGACACTTTTGCTTTCATTGTAATAGAGGGAAAGGTGCTAAAAAAAAAAAAAA
19321 AAAGGCCAAATAACATTTTATCCTATATGACACTCCGTCTTTTTGAAAGTGCTCTTAAAT
19381 CATGAAGGTGTAGAAATATGACGTACTAATAAGGTTAGGCATATTAATACATCACGGCTC
19441 AGTAATCTGGCCTAGAGAATTCCATGGACTGTATAGTCCAAGGGGTCGCAGAGAGTCGGA
19501 CACGACTGAGCGACCTTCATTTCACTTCACTCAATTCAGTCTCAAGAAAAAATATCGCAC
19561 CCTTGGGATTCCACTTCCAGCTCCTCCCTACCTCCTCCCGTTCCACCTTGGATCTCCTCC
19621 TTCCCTCCCCACTACTTCTCCGCCTTTCCCCCGTCTCCTCCTTCACTATCTGCTCCTCCC
19681 AGCGCTTGTCGGGAGGTTTCCTCGCGTGACTACGTCATGAGGGAGACGCACTGCAGCACC
19741 CGGGGCTGACTCTTTCCTCTCGTCTCTATGATTCTCTTTGTGACCGGCCGGACGGCTCCG
19801 GACTCCCAGAGTTCCGTTCCAATAGTCCTCCGGGCCGACAGGGGGACGCCGCGGGTGGGG
19861 TCGGGATGGGGGGGGTGGGGAACAAAACAAAACTGACGTTCTCGCGAGAGGGGAGGAGTT
19921 CTCTCCAATCTTTGAGCGCGTGAGCGCCTGCGCGCTCCCCGCGGTGCGCGGTAGCTGGGA
19981 AAGGGAAACGAGGAGCCCGGCTGGACTCCCGGGGAGATCGCGCGTGCGCTGTCGCGAGCT
20041 CGGCGCTGCCGGGGCGGCTGAGTTGGGAGCCGGCTGGTGTGTGTGTGAGAGCGCCGTGCG
20101 GGACGCGGGGCA***ATG*** ***TTCCGAAAGGCCCGGCGGGTGAACGTGCGCAAGCGGAATGACTCG***
20161 ***GAGGAGGAGGAGCGAGAGCGCGATGAGGAGCAGGAGCCCCCACCGTTGCTGCCGCCGCCG***
20221 ***CCTGGCACCGGCGAGGAGCCTGGCCCCGGCGGCGGCGGAGGCGACAGGGCCCCCGCGGGC***
20281 ***GAGTCCCTGCTGGGCCCGGGGCCGCCGCCGCCGCCGCCGCCGGCCGCGCTGACCTCG***
20341 ***GGGACAGAGGCCGGGGGCTGCCTCCCCGGCGGCTTGGAGCCCGGCAACGGGCTGAAGCCG***
20401 ***CGCAAGAGGCCGCGCGAGAACAAAGAGGTGCCCCGGGCCAGCCTGCTCAGCTTCCAGGAC***
20461 ***GAGGACGAAG***GTAAACCGCCGCGCCTCGGGGGGCCCCCCGGACCYCGGGATCTCTATCCT
20521 AGCTAGGCCTCCACCGCGGGTCCCTGCGTTCCCAGGACGCTCCCAGCCTCAGCATCCCCG
20581 CCCCCCGCCCCGGGAACCCAGTCGCTTCTTCCCGCCCCGACCCGCTGCACGTCTTCCCCC
20641 GCCTCCGGCCTTCTGCGCAGGCAGGGTGGTGGGGGGGGCGGTGGGGGGGATGTGTTCCCG
20701 CGCCGCCGCCGGCCTTCTCTTAACTACCGCCTCCTCCTCGCCGGCCTCCCGAGCATCTCC
20761 TGTCCCGAGGGGATGGGGACCTAAGATCAGTCATTCTGAGTTTTTTTTCACCTGGAAATT
20821 CAGTGTCTCCTTGTCCTGCCTCGTTCCTAGCCGCTGTGTTTTAGGGAGAACCAGACTGCT
20881 CTATTAGATGGCTCCCTGGACTGCCCTGGGCACGTTCTCCGGGCCTTCCGTAGGTCCCCG
20941 TGCCCACGCCTCTTGGTATGCTCGCCCTGGTCAGACATCCTTGAGTAGGAAGGGGCCAGA
21001 CATCCTTGACCAGAAAGGCTTAGTGTGTCAAGAGGAGTGCTAGGTCTGGAAAGAGAAGCC
21061 TATAGTTGAAGCTCTGGTTCCGAGTCACTTTGGGCCAGCCCTTGGCGATCGCCGATCCTG
21121 TTTCCGATCCTCAGTTTCCCTGTAAAAGCAAATAGGGGTACAAATGTGCGACCTTAACTC
21181 GCATTAACATTTGGATGGTGAAAAGAGACGGTGTAATGGTAATTCAAGGCGGTTTATCCA
21241 ATCTGGTGTGAGGGGCCAGGCTACAGTGGATTTAGATTTCTGATCGTAGATGAGCCTTCT
21301 ATCATTTTACTTCCCCAATTGTTGAGTGTATGTTTGTATAATCCAGTTAAGTCTATGTTA
21361 TGAAAAAAGAATTTAAGACTTTTGGAAATCTGACTTGCATCTTGATGCGCGATGTTTTCT
21421 TGTTTCCAGTAATAGGCAGTTTCTGAAGTTTGCCAGGAGTACAGTCACATTTTCGTTTTC

Appendix B. Continued

21481 GCTGATGTAGGGACAGCTTGGTAAAGTGTTTGCATTGACGTTTAAAGTTTTCAAATCAAA
21541 GATGATGGATACACCATCGAAATTTATTTTTGAAAAGTTGATTAGCTCTCAGCTTGGTCT
21601 TGAAATAACTGTGTCCGTGTGTGATGGGTTATAGAGTTACTCAGTCTTGACAGGTCTGTA
21661 AACTAGCACCTTTTGATTTTGGATATTTTATTTAAGATACCTTAAGAATTTGATAATTAG
21721 GTGCTTTTTTGAAGATCTCTTTTCCTCTCCTGAAATAAATGATTTAAGAAAGACAGTAGA
21781 ACACAGAACTTTGTGCAGTTACATGTGTTATGCCTTTAAAAAGTCTCAGATTGGCATTTT
21841 ATTCCAGATTATGAAGTTGATTCAGTTTTTAGAAGCCGATTACGGATTTTAAGTAATTTC
21901 AACTTGTGTAATGATTTGGAATGTTTGAGTATATTTAGAAGAATTCAAAAGTTAGAATGG
21961 AAAATATTGAATGAAAATAGTTGCTACAGATTTTGTTTATTTTTTAAAG***AAAATGAAGAA***
22021 ***GTTTTCAAAGTGAAGAAATCAAGTTACAGCAAAAAGATAGTAAAATTGCTTAAGAAAGAA***
22081 ***TATAAAGAAGATCTTGAAAAATCTAAGATTAAGACAGAACTCAACTCATCTGCCGACA***GT
22141 AAGTACTAATTTGATTCTTTTGAAGTGACAAAATGAAACTCTCACAGGGATTGAATAGAT
22201 TTGGTGTATATACAGTGAACTTGTTCGCTTGATTCTGTCATTGTCAAGGGCTCAAGTTGA
22261 GTAATGATGCAGTTTTATTTAATTCCAACATGACTGCATTGTGAATGCATTATTTAGAAA
22321 GACAGAACCATTGCCACTGGAATGTTTCTGCTGCAGTGACTAAATTCTTCCAGTTTTCTG
22381 ACTAGTGTTCCAAGTGCTGTTGAACAGAGAGAGGTTTCCCACTTAAACCTGAGACACAGT
22441 TTGTTTTTGTTTGAAGGAGTACTGTGCAAATTCCTGTACTTGTATTCTGTACTTATATTT
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22561 AATGTTTAGGAACCCCCTAAGTCTAATGTTGATAATGTTCCCATTTAGCAGCCTTATCAG
22621 ATACATGAATGCTCACTCCTGGGTGTGTGGCACCTGCCTACATTGTACAAACCATCTCTT
22681 ATGTCTTCTCACAGACTAAGTTAAACTTTTAAGCATTTATTTGTCCTTAGGAAAGTGGTT
22741 TCATGATAATATGCTGTGGTCTAGTTATTCTCACGCAAATGCTTTAGGGAGATCTTTTTA
22801 TTTTGTGGAATTAAAGATAGAAATTATTATTGCCCAGTTCTCCACTATAGGCTTGATGTG
22861 GGAACGTATGTCATTATATCTCAAGTTTGCAATCTAATTTTGGAGACAACATCACTTCAG
22921 TGTATAACTGTATCCCGAATTTGGCAAAATGTCTAGTGAGTGACTTAAAGCTTAACTGCA
22981 CTTTTCTTTTTAGAGATGGGTGAAGGAATGCTTCATAGATAAAGTGGAATTTGAAGCTGG
23041 ATTTTTGATCTTTTAAATTGCAAAGAAAATAGGGGAGGTGTGGGCCTATTATTCAGAGAC
23101 TTGCTGTTTTGGAAAAGAGCAAGTACATGTGACAGGCTGAATAGATGATGTGTGGGGGAT
23161 GCAGAGGTTTAGGTGAAAGGTACGTTGTTGACAGATTGTGAAGGGTGTTTAGTATCTAGT
23221 ATGAGGTTTAGAGTTGCCATTTCAGGGGCCTGCCAGGATGGTGACATGATAAATGCCTTT
23281 TGAAATCTTAACCAGGTGTTAGCATGCAGCATGGATTGGCACAGGAAACATTAGAGGCCT
23341 AAAGCAGTTTAAATGCAAGGGCCAGCTTTGATCCAGTAAGAGTGGGTTTAAAAAGGTCTC
23401 AGTTGAGCAAGACTGAGGCTTTTCCTGACTCAGTGGGAAATTGTAGGTGAATTTAAAATG
23461 TTTGCTGTGGGTGTGGGGAAAGGAATAGAAAAAGATTATTTAAGAAGTATTCCAAAGGAA
23521 GAATTGGTAGACGTAATGTATGCTTCAGCTATTGGATCTAAAAGAAATAGATTTGAGCTT
23581 TTGTGCCAGGTGACTGAGAGGATAATTATGTATGATATACAGAAATAGGGAATGCAGGTG
23641 GGAGGAGGAGTATTTTTAGGGGAACTGATTATTTTGGAGTCTAGGAAGCTGCTTCATTTT
23701 TTCCAGATCTCTTAAGAACCAACTGTGTATGATGAAATTGAAGAAGAAAGCTCAGGAAAA
23761 GTGAGAACTGAGAAAAGACCATTTACTGTTGGGTTTAAAAATTGAAGCATGTGCAAAGTT
23821 TATGTGGCCACCTTGTTTTCATATTAACTGATTTCCTACTTAGCAGATAACTCAATTAAT
23881 ATCCTCCCCAAAGGTAGAGGTCGCTGTTGCTGTTTCATTAATCAGCTCACAATCCCAAAC
23941 TTTTCCTACCCCCAACCCTCCTTAGATAGGCAGAGGTGCATTAACTGGAAAACAAGGGTA
24001 GTATCAACTTAGAAAACCTACCTTCGGGATATTCCAAATTGATGAGTTCACCTTTGAACT
24061 TGAATTTGGGTAGAAATAGCCAGTGAATAGTTGGAGATGTAGATTTTGTTTTGGTGTTCA
24121 TTGAGGTGATGATTGAAGCCAGGGGTAAATGGAATCGAGGCTGCAGCATAAAGGAAATAT
24181 TCCTGAGGACCAAAGACTTCACCTGTAATAAGTGTACACATAGAGAGGATGGTTTACTCA
24241 AGAGATACAAAGAGTCATAAAGTCCAAAGGAGAACAGATAGGGAAGTTTCAAGATGATGA
24301 GTGTTGATGAGTGTCAGTTGTATGAAATAGTATCAGGAAGGTCAGGAAAAGGAAGGGCTG
24361 TTGGTGTGGTGGCTGGGTTTTGTGGGGAGCGGGGGAGGCATCTGGCAAAAGAGACTAATT
24421 TACTTGCCAAGCTCTGCCTGTTTAGGGGAAGGAACATGGTTTGCTAAATTGCTCAGTGAT
24481 ACTGTATCAGTTAGCTGTGGCTGCAGCCTCTGCTTTAGTTGGTGAGCTTGGAATGGGAGA
24541 TATCAAAGACTGTTTGCCAAGGTCGAGACTGGCAAACTGGTGAGGAAGTGGGAGGTTGCA

Appendix B. Continued

24601 GGTCCACATGGCTGCTTTGGAACATTTGGCAGAGAGTAGATGGCAATAGCTAGAGAAATA
24661 GTTGGGATCATGTTGAAGTTCTTTTGCTAAAGAGAAGCCTGTGTCGCTTTGTAGACAGAA
24721 TAGAGGAGATGGAGAACGTGCCCACAGTCAGAGGAGGATATTTGTGGCCTCAGGTTTCCT
24781 AAAGTAGCGAAGACATTGAGGCCCAAGAGGAGCCTCAGAAAAGGTTATTCAGCCTCTCTT
24841 TTTATAAAGCTGTCTGGAAGGATAATACAGGACTAACAAAAATTCACCTGTCACCATGTT
24901 CTGGACTTTGTATTTGCTCTGTCACAAGCAAATAACTTATGAACTTACTACAACACTGAG
24961 TCTGCAGGAATCATCTGGGCATACTGGATCAAGTAAACCTTTTCGCAATTCTGTTTGTCT
25021 GTTAAAAGCCATTGATTTAGTGTTAATTGAGAGCAAAAGCAAATGGGTAGTTCTTGACTC
25081 CCGGTATCTTTATGCATCTGATAAAGTGAGTTTTAAAATCAGCCTCAGAATGCACTTCAG
25141 AGGGAATCTGTGGCAATGAGGTAGAGTGGTGAATTAAAAAAATTTAAGAGATCTGAAGCA
25201 GAGTGACCTAAATCTTCTAAGTAAAGTTGTGGGGCAGGGGGTGAGGGGGGGTAGGATCTT
25261 ATCTCCAGGATTCTTCTTTAGAGGAGTAGGAGATGTAGGAGATGGTTTGAGAACGTTCGA
25321 AGCAGTTTTGGGGAGGGGCACATGCTAGGGAAACAACAGAAGTGTATCTTTCATATTTAG
25381 AATGAACTTTTTGTTTCACTCTTTCATTATATTCTTGGTGCCTCGTATGTGCATTGCCAG
25441 GTTCAGTGATTGAGGGCGAAAAATGCCTGTTTTTCTGCATTTTGCCTGGGGTGGAAGGTG
25501 TAATAGGACTATATAGTTATAAGTAAAGTAAACCATTTGGTATAAACCTCTGTGACAGTG
25561 GTGCCCCCTGGAACCCTGTGGCAGGAAAGTAGTCTGAAGCTGGCCTCCGGAATGGCCTGC
25621 GGAAGAGAGAGCTCTCTTCTCTGTCCATGTGATGGGGTGTAGCGACTCCACAGCTGCCTT
25681 CCTGGTTTGCATGCGGTGGTTTTTAGTGCTAACTAGTTTCTAACTTGTCTCTGTGTCCCA
25741 GTTCCAGGTTGCCAGAAAAATCAGGTTTAGTCACAAATCCACTGTTCCAAACATTCAGTT
25801 GTGGCAACAGCCTCAGGGGTTTACACAATGTAACTGTGACTTTTGGGTCTCCACTCTTCA
25861 GATGATGGTTATAACCTAAGACAATATCCTATAAAGTGTTCATTACAGAGAAGTTTCTAT
25921 CTTGTTCTTGATAGAAAGCTGCCTCAAATGGGCTTCCCTGGTGATTCAGACGTTAAAGAA
25981 TCTGCAATGCAGGAGACCCAGGTTTGATCCCTGGATCCGGAAGATCCCCTGGAGAAGGAA
26041 ATGGCAACCCACTCCAGTATTCTTGCCTGGAGAATCTCATGGATAGAGGAGCCTCGGGGG
26101 CTGTAGTCCATGGGGGTCTCACAGAGTTGGACATGACTGAGAGACTAACACAGATGGATT
26161 TAGAGTTTGATGAAATACTGACATATAAAGTGAATAATGCTCCCAAGAGGTGAGAACAGT
26221 TACCTATGAGAGTTGAGAGGAAGAAGTTAGTTTTGGTAAAGTGGTAAACTTTCAATAACG
26281 TTAACTAAAGGCAGGGATTGGGCAGGGCCATGCGTCCCTCTACCCTCTGTATTGTTTCTG
26341 CCTCCTGGCTGGCTACTGTGATCATGTCACATGTCAGAAGTTTGAGGATTTTTATAAACA
26401 ATTAGAATAGTTGATGCTTTAATGAATTGAGCCTATAATTGCATTAGAAGTGCCTCCTTA
26461 ATTTATGGATTTTGTTTTAGATATGTTGTATTATTTATTTATTTTTTCCAAAAATGTAG***A***
26521 ***CGAACCACCTTTGGACAAAGCAGGTCATGTTAAGGACACCAGTCTAGAAGATGGAGTTAT***
26581 ***CATCAGTGAACACGGGGAAGATGAAATGGATATGGAAAGTGAGAAGGAAGAAGAAAAGCC***
26641 ***AAAGGCTGGTGGAGCCTTTTCAAATGCTTTATCTTCTTTAAATGTTCTCCGTCCAG***GTAT
26701 GTACTTGCAGATGTTCCTCACAACCGAAGTGATCCCTCAGGAATGAGCCATCTGTAATGA
26761 TGGTCGTTAAGGAAGGAGTTAGCTAAGTGGTGTTTCTTCCGACATCTGTTTGGAGCCAGG
26821 CTGTTTCCTTTTGCAAATACAGAAAGAAGGAGAACTAGAAAGTTCTCAAGTTCATTCTCA
26881 GACAGGTAGTTTTATTATCAAAACAAATGAGGGGTTCAGGTAAATGGATAAATAGTACAA
26941 AAGTCCTTTTTTTAAAAATGTATTTCAGATTAAGATTTTAACTTTTTCAAGAACTGCTAC
27001 CAAAAGCAAAACTGCTGCTAGCTTTATATTCAGGGTTTTTTTTTACATTTTAATGGAAAT
27061 AGTTTGCAGATCTATTCACTGGCTAATCAGCTTTTGAATAAATACTTAGTCACCAGATTT
27121 AAATTATGAAGAGTAAGCATTTAACTCATTATAAAATTTAAAAATGCTTACTCTGTGGGA
27181 ACGCCTTAGAGCTTTCACTGCTGTGACCAAGGTTTGATCACTGGTCTGAAAACTTCCACA
27241 AGCCGTGCAACACAGCCAAGAAACTGATAATAATTTTTTTAAAAAGTCAAAATGTTTGCT
27301 CTGGGTTTGTAAAGTTGTGAAAAATTACATACATAGATCTAATAGCATTCTGATTGGTAC
27361 AGTCTTTATGGGAAGCTGTTTGACATTATGAATCAGGAGATTTCAGATGTGCTTACTTTT
27421 TGACCTGTAATTCCATAGCTATAAATGTATCTGATGGAAATAATGTTTTTATTGTAGCAT
27481 TAACTGCAATGAGAAACATTTAGAAAAAATGTTTAGTAAGAGAGGAATAGTTTAGTAAGT
27541 TAAGTTACGGTGCATTTACATGATGAGATATCCCATAGCTACTTAAATAACAAATTAGAA
27601 AAGTAGCAAGGCGTTAATTTTTACTGTAGGTAAAAGTGTTAATTTGATTATGTAGTAGAT
27661 TATGCTTTATCTATTGAATGTTGTAAACCACTAAAAATTGTTTATGCAAAGCAACATGGA

Appendix B. Continued

27721 AAAGCTTATATTTTAATATGAAGAAAAATTAGACACAGGATTATATATTCAATGTGTTTT
27781 CTGGTGACCTGAAAACTATTTGCAGGTGAAATACACTCTAAATTGAACAGTGTGTTTTAG
27841 GTTGTAAGAGTGCAGATCCCTTTTTTGTTTTTGTTCTCCAGTTTAGTAATCAATATGACT
27901 TGATTACTTTTGTTTAGTTCTTAAAAATGAAAAATAAAAACCATTAAAATAAAACTGTTT
27961 TCATGAATTTTCCCCTCCCTGCCTTCCTGTATATATTGCCATTGTATATATTTTGTTTCC
28021 TTAGCTTCTTTCAGTTTCTGTTTAAACTAAGTTAAAAAAATAAATCAGTCGTACAGCAGG
28081 CAGAGTTTGGGGAGAGGAGCGGGGATGCCAGCAAGGGATGTTAGAACATCATTTATGCAC
28141 GGCTGTCTTGGAGAGATTCTTTTATCTTTCATAGTTTCAAGAAAATAGAACCTTTCTTCC
28201 TAATATGGCATGGGTTCTAATCCTTACTGTTTGTTTCCAG***GAGAAATTCCAGATGCAGCT***
28261 ***TTTATACATGCGGCAAGGAAAAAGCGTCAGATGGCCCGGGAATTGGGAGATTTCACTCCT***
28321 ***CATGATAGTGAGCCTGGGAAAGGCCGCCTTGTTAGAGAAGATGAGAACGATGCCAGCGAT***
28381 ***GATGAAGATGATGATGAGAAACGCCGCATAGTTTTTTCTGTGAAAGAAAAGTCACAGAGA***
28441 ***CAGAAAATTGCAGAGGAAATAG***GTAACTTTATTTAGTGCAGTAGTAAGCCTGTTGTTACT
28501 AAATTTTATTTGATACTACAATCAAGTAAAATGTATCACCTTTATTTAAACTATGTTTAA
28561 TGTTGAAGATTCCCTCATAGCTCAGTTGGCAAAGAACTTGCCTGCAATGCAGGAGAGCCT
28621 GGTTCGATTCCTGGGTCAGGAAGATTCCCCTGGGGAAGGGATAGGCCACCCACTCCAGTA
28681 TTCTTGGGCTTCCCTGGTGGCTCAGCTGGTAAAGGATCCATCTGCAATGCAGGAGACCTG
28741 AGTTCAGTCCCTGGGTTGGGACGACGATACCTTGGAGAAGGGAAAGGCCACCCACTCCAG
28801 TATTCTACCCTGGAGAATTCCATGGCTCATGGGGTTGCAAAGTGTCGGAGATGACTGAGC
28861 GACTTTCACTTCACTTTCAATGTTGGAGAACTGTTCTTGCTTGGATATTAAGTGTCTTAC
28921 TGGAATTTGGGGGGAAGTGTAGCATGATTTTGATAGCTAATGTTTACTGAGGACTGTGTG
28981 ACGGGTATTGTGCTAAGCACCCAACATGTTATAAATAGATTTAATTGTCTCTTCATTCTG
29041 AGGAGGCATTAGGTTTCATTCTTCTCTTTGTTGAATTGATGTCAAACTAACAGAGGTTAG
29101 GAAGCCTGTTTAAGGTTTTAACTGTTAAATTGATACTGCCTCTTGCTTAAGGAATAACTG
29161 TGTATTAAGACAAGTAAAGCAAAAGAGTCAGATATGAGACAGATCATAACAGACACTTTA
29221 ATTTTAGTAAAGTTGAGTTCATGCCAATATTCTTGCTTGATTTTGTGAAATATCAGGGTA
29281 GCATCTGTTCCCTTGCTGCTTTTTGGACTTATTTTCTCCCTGCTTATGGCCATAG***GTATC***
29341 ***GAGGGGAGTGATGATGACGCTCTGGTAACTGGGGAGCAGGATGAAGAGCTCAGCCGATGG***
29401 ***GAACAGGAGCAGATAAGGAAAGGAATCAATATCCCGCAG***GTGAGAGCTAAACAGCTAAGC
29461 TTCAAACCTGAAAAAGTGAATTAAGATTTCTAGCCTGTTTTATTGAAATGAAAAAACATA
29521 TTGCCAAAAAAAAAAAGCTGTCCACTGTATAGATTAAATGTTTCTTTCTTGCACAACTGT
29581 TTTTTTCCTGACTTTAAAAATTATTTTGTAGAAGATGGATTTCTTAGTAGTGCAAAAATC
29641 TTTAACTGTAGATGAAAAATATCTTCCAAAGTAGTTATAACAACTTGTTCTATAGAAGTA
29701 AGTAGTTCAATGGAAATGTCATACAAAGTTTCTGTTAGTGTTTTGACACAATTTAACGTC
29761 CCTACTAACTCAAAATCTCTTCATTATTGTTTTCCTGAAAGTTATTGGAAATGAATCCAG
29821 TAATCCCATAAGAGAAATTTCATCCAGCCCTACTTTTGATTTTGAGAAGCTCTGCAATTA
29881 CTTACTTTAATTACATACTGTTTAAAAAAAATGGGTTTATTATTTTCTGTTAGTTTTTTG
29941 TTCTTTTTGTAGTTTTAGCTACTGGTTTTAATATATATCCCATTTCTCATCTAG***GTTCAA***
30001 ***GCAAGTCAGCCCACAGAAGTGAACATGTACTACCAGAACACCTACCAGACAATGCCTTAT***
30061 ***GGTTCATCCTATGGCATTCCGTATAGTTATTCAGCCTATGGATCATCCGATGCCAAATCT***
30121 ***CAAAAAACAGATAATACAGTCCCTTTCAAAACTCCCAGTAATGAGATGACTCCCGTTACT***
30181 ***ATTGATTTGGTAAAGAAACAGCTTAAAGACAG***GTAGGGCAGATCAACTTCTTAAAGACCT
30241 GTCTTCTAGCTTTCCATTCCCTCAGGCAACATGTAGTCTGGGGTAGCAGATACAGAATAT
30301 CGGCAAAGAAGATTGACTGGCCTATCTTCAAAATCTAACCAGAAGATGACTTCATACTTT
30361 GTTCTGCTACCTACTTGGATTCCTTCTGTGGAAGCCTCGAAGTTTTTTTTTTAAATACCA
30421 CTCCATGATCTGGTTGAAGACAATTCTCAGTTTTCTCCTCTTGGACTCTGAAAAAAGTGT
30481 AGTCAGGATATGGGGGGAAAGGCAAAACAGTGAGATTTTGGTTAGCCCACCAAATAACTT
30541 TATATAAGAAGTCTGTAGACTGCCATTTTCTTGAGCAAAACAGGAGTACAGATGAAAAGA
30601 TCTACTCCAAGCATTTTTATATTCTAATAGCGGAACGAAATGCATTATTATAAATATTGC
30661 AAGTGTCCTGACAGCATAGACTTCCAGATGTAAATGTGTGATATTTATCCTTTTCTGTTC
30721 TTAAG***GTTGGACTCCATGAAAGAACTGCACAAAACAAACCGACAGCAGCACGAGAAACAC***
30781 ***CTGCAAAGCCGCGCGGACTCCACCAGGGCCATTGAGAGATTAGAAGGGTCTTCTGGGGGT***

Appendix B. Continued

30841 ***ATTGGTGAACGGTATAAATTTTTGCAAGAAATGCGAGGGTATGTCCAAGACTTGCTTGAG***
30901 ***TGTTTCAGTGAAAAG***GTAAGAATGCAAAAATATTAATCTCTTTGAATAAGGCAAAACCTA
30961 GGGAGGTCTAGATTTCTGTATTTCTCAGAGAATATCCACTGACTTTAGCTTTAGAAGTGA
31021 TGAAAAGTCATGCTTCATTTTCTCCACTTCATTAGCAGCATTCTGTCTTGGGGGCAATTT
31081 AGTTAGTGCCTTTGTTGAAAACTAAAAAGATAAATCAAAGCACATTTAAAGACTTTGGCA
31141 GATCCTGCCATAACTTAATGATGGATTACCAGCCTGTCCTGGGTTATTAGAATATAAGGG
31201 ACTCACTGATGAAATTTTCTTTCTTATGGTTCAGATCTAAACTTATCTTTAAACTGTTTG
31261 TATAGGTAATTCCAAATATGTATAGCATAAAGTTGTAAAATTTTATTAGTTTTTATTTTT
31321 TACCCTAGATAAAGTTGATTGCCATTGCTAGTACTAGTTTTCATCTGAATAAGCCTGTGC
31381 TTTATCCATTTCAAAAACCATTCATTGACCATAATTTGTTTTAAGGTCTCATTTCCGAGT
31441 TTTTTTCTTTTTGGTATGATAGTCAATTGGCAGTTATTTGCGTTCTGTTGTACCATTTGT
31501 TTGCATTTTTTGTTACCTGCACACACAATTCTCTTTCAAGCAACTTTTAATTGATTTTTC
31561 TTTAAGACAGATCTATTGTTTGATGCACTGGTTAAAAAAGGCATTCGTGGACTTCACAGC
31621 CTTGTCTTTAAAAAGAATTAAACTCATAGTACTTTTGAGTATCCTGTCTCTAAACTTTCA
31681 TGGGATACAGGATTAGAAGGCACATTCTCTCTTATCCTTGGCCATATGAATTAGCATAGG
31741 ATTTGTAGGCATATAGTATGTTTAAATGAGTATTTTAACTTTTTATTATCTCTTGATCAG
31801 TATCTTAAGGCCAAAGACTGGCTAAAGAACGGTGCCAAACTGTGTCTTCCTAATGAGTCT
31861 TAGTGATTCCAGCCAGTCTGGTCAGATCCATGAAGTCTTCTCTGGATGATTTGTGTTCTT
31921 GTCCTGATCATCTGATTTCTGGAGAAGCAGCTACATTCTGGGCTGTAATTGCCCGTGTGG
31981 CAGGTCTGTGGCTTAGGGTGATAAGTACCAGGGTGAGAAGGAAACATAAGTTCGACTGTG
32041 TGCAGAGGAGCTGAGTTAAGCGGGAAAGTGGACAGAAATGTCAGAGCTGGCGGTGTGTCC
32101 TACTGACAAAGCCCTTGTGAACCCAGGGGGCATGGTGGTGGGAGCTGGCCCAGTAAGCTT
32161 TTGTGACTAGAGAAAGGATCTTTGTCTTAAACCATACTTTGTAAGTAAAATTTGTCCCTG
32221 TGGAGTTTTTCTTGGAAATATCTTGGTCTTTGCTTTGCAACTTAATTGAGTAAACTTAAA
32281 CACTGTGATTGCAAACTGAGCTCTTAAAAAATATTGACAGTAATACGATCAACTAGATTG
32341 TACCACTCTTGAGTTAGAGACTAGTAGAATATCTAGTTCATATTGTTTTCAGTTGCTTAT
32401 TATCTTGAAAACATTTAGTAGTATGCTTTCTCTCTCCTGGTTACTCTGTTCCTAGGGAAG
32461 TATACCTCAGACTATGCTGAGGTTTTTACAGAGTCACTTAGGTACATGTTTTTGAAGCAG
32521 ATCTTAAACCATATCTGTTATATAGTGGCTACATTTTAATTAAAAGAGAAGTAGGTAATT
32581 CCCTTTTGAAGATAACTTTATTTCGTTTTGACATTTCCATATCCATTTGATTTTACGGAG
32641 CCTGCTGCTGAGTTAATAATGTCATAAATTGTCATTCTGGAAATACTGTGTCAGGGTAAG
32701 ACCATAAAAAGATAAGCTTTCTTTTGTTTTTTTCTCTTTCATTATAAAGGTTTAAGTGAT
32761 TTCAAGCTTAGATTTTTATTTTTGCAGATTATTTTAACTTTCCATTGTTTTGTTTTCCTG
32821 GAGTCGTCTTCCTTCTTGTGTTCTTTGCCTTAAATTTGGTCTTGAAATAATGGTAGTCAG
32881 TAGCAGTTTTGAGTTTGGAGAGGGTAAAGAAATTGTCACGGTGCACTCTTGTAATAAATC
32941 ATAAATCAATATTGTTAAGAAACATGAAAGAGAGGTGAAAATGTGCTTTAGGACTTTTAT
33001 TCTCAGAATTTTCTCAGGGCTTTGTTAAAAGGCATTTTAATTACCTAAACAAAAATTACA
33061 TTATCAGAATTTTAACTGTAAATTGGATAATTAAGGCTCTGCCTGATGCAGCCTCAGGCA
33121 TCTAGCTCTTGGCAGTAAGAAGAGTGTTAATAGCCTCCAGGACTAAAGGAGTGCATGCAG
33181 GCACATAGGTTCATTCGTCCTTAGTCCTGTATGGTTTGCAAAAAAATGAGGGGTGGGTGA
33241 GTGTAAGTCACAACAATATGGCAATAATTGCTTGGAAAATGGATTATGCTTAATCTTCAA
33301 GTTAGAGTCTGTGGGCTCAGCTGATATCATCAAGGTCTGGGGATGCTGCTGCCCATGGTG
33361 TAGCATATGAAAGAATGAAATAACTGCATCAGTTTGACCTTTCTGTGAACATTTGTTGCT
33421 TTTGCAATGGAATCATTCAAGTGTATGAAATTGCTTTTATTCCACCTGCTTCTTTTTATT
33481 TTCCATTTGCAGTCTGTTCAGTTCAGGAAGCTGCTATAACACTTGGTGCCAGTGTTGCCC
33541 AACTCTAAATAGTGTGTTTTTTTATTCGCACTAACCATCAGGAAACTAGAATTTCTGGTT
33601 TCCTCAGGTATGTCTAGTGGTTGTTAGTGTTTTGTGAAAGTTTCATAGCATTTGATTATA
33661 TAGCATAAGGTATTTATAAAAAGAGAATGTTTTCTGGTTTTTCCTTTTAAGTTAGGTCTC
33721 ATTCCATAAGCTTTTTGTATTCTGTATGAACCAATTGAATGTTTAGCTGTGGCTGTTAAC
33781 CTGGAAAAATCTGTTGACTTTGTAAAGCAACAATTTATGCAAGCAATTTTATTTAAAAAT
33841 ATGGAAAAAATTAAATATTTGATGGGAACAGAAGAACTTTCTGAGTTTCAAATAGATTGA
33901 TTTTTAGACAGGTTAATGATAGCTAGAAATTTTATTCACTTTATTAATATAAATGAATGC

Appendix B. Continued

33961 AGTTCACATTTTAAATGTGTAATAAAGCCACAAAGTAGTATAACATTACTGTAAAGTACT
34021 TGGTCCTTGTGAACGATAGAGCAATTGAAATGTCCAAAATTGGGCAGTTAGCAAACTCTT
34081 TACCTACTCAGAGCCGTTTTTTTGGTCTTACACAGCAATATTAGTTTATTTTAAGCTACA
34141 AAAATTGCAAGGTAGATCATGCTGCTAGAACACTAAAAATGGATTCACGTACTTTAAAAG
34201 AAAAGCACTAAAATTTCTGACATTTCTTTACTCACTCTCTTTACTATTGTTAAAGTGTTT
34261 AAGTTTCCTAACTAAACTCTTGCTCCAACAACTAGGCACTGGACATTATAGTTCCATATA
34321 CGGAATAAAAGAAGAAGTGTTACTTCCCAGACGTCGTAGAACTGAAGTACTATTTGTATT
34381 TCATTGGAAATTCATGATTATTCTGTCCAGAGGGTACTCCTTCAGAAATCCGGAAATAAT
34441 ATGGAACCCTCAAATAAATAAGCATCTGAGGAGTTTGGTGTTACGGAGTGTGTTGTATTT
34501 TTACAG***GTGCCACTGATTAATGAACTTGAATCAGCAATACATCAGCTGTACAAACAGCGA***
34561 ***GCTTCCCGCCTTGTCCAAAGACGACAAGATGATATTAAAGATGAATCTTCGGAGTTTTCA***
34621 ***AGCCATTCAA***GTCAGTCCATCTTGAAGGTTTTTATTATTCATTAAACAACCTTGTTTACT
34681 TAGTACATCCTAATTGAAACGTAGACATCTCAGGGCATTTTTTTTTTTTTTAAAGAAACA
34741 AATTTCCTTGCTCTTATGTCAGGTAGATCTAAAATTATTAATGGCATGTGCTTGGATTGT
34801 GTTTAAGAAAATATAGCTTCACAAAGGAGTGCAAATCATGTACTAATATTCTGTCTTATT
34861 CTTCTTTGGGCATATTTTTTCATTCTTCAAAGTATAGTAAGGACATCATCAGTATTTTAT
34921 CTTAAAACCAGTATCTACTTGTCCCTAAGTGATATTTTTTCCCCCATTTTAACATCAATA
34981 CAAACGCTAACACAAATGCTGTTGTTGATCATAAGGTTTTGAGTTAGGGATTGGGAAATT
35041 TTGGATGGGAGAGAGGGTTTTATTACATAACAAATGATACAATTGCAAGATTAGGGGAGT
35101 TTTGAGCTTCACCCCTTGCATAAAGCAAAATTTTTTGTATTGAAATTTTTTTTTTTTTTT
35161 CCTGTTTTGCCTTATATAACTAGGTTTTTTATTTTTGGGGTTTTTTTTAGTAAGTCAGTT
35221 GATGCCAACACTTCTTGAAATTAGAGTCTAAAAATCTGGAAATTTTAAATTTATATTCCT
35281 TTCATTGTGATGTGGGTTATGGAAAACCAAAATGAATTAGAAACCTCTTAATAAGAGTTT
35341 AGCTGAAACTCTTTAACCAGACATATTAAAAAACATTTTAATTCCACTTTTTCATTGCCC
35401 TCTATATTCTTGAGTACTGTAATAGGAATTTTCCTATGTTGGATTTCTCCTGGCTTTTTT
35461 TGTCATCTCTTTGGTCCCTGTGTCAGCAGTTCTTAAATGGATGTATACATCAGAATTACC
35521 CATGATATTAATTTAAAATGCAGATTTCTTGGTCCTGTCTCCAGAAATTCTTGCCAGGTC
35581 TTGGATCGTGTCCAGTAGTCTGCATGTTAAACAGCCACTCCAGGTGTTCCAAGTGCAAAG
35641 TGCCCTGTTCTCATTCTTTCTGAAAACAATGCTTTGTCATATAGTGTGTCCTCTAAATTA
35701 GAGAGACATTGAATCCCTGGAAAGCATTCTCCTGTGTGCCGAGTGGCGTTTTGGAGCATC
35761 ATAAAGAGACGGTGACATGGCCCTCAGATGAGTCAGACTCAGTGTAAGCCATGTGAATGG
35821 CTTTGACCTTCTGTTTTTGCCATACACATTTAAATTTGGATTAGTGCCATTTGGGGCACC
35881 TAAATAACTAATGCATGCCTCTCAAATCACTTTTGATCCAGACAGAACCAGATTTCTGGG
35941 GCAGATTACAGGTAATCATTATATCTATGATGGGAAGTAATTTCCTTTTAAGTTAAATGG
36001 TTGTGTGAGCTGTGCATTTCATCAAATACCTTTTCCTAGCTATAATCTGTTCTTTATAAT
36061 TTGGAGGTATTTACTTACATGGGATTCAGAGTAAGGGTTGAATGATGGGTGTTAATTATC
36121 AATAGATTGACAGTTGGAAAGAAATGAAAGGTAATGAGAAAGTGAAAAGTATTGTCCTAA
36181 AGGGAGTATTCATTATTGCCAAAGATTTGAGGAAGATTGCATGGGATACTGAATTATCGA
36241 TTTCTTTTTCTGATATCTTTTTTTTAAAGAAACAAAAATACAGCCTGAAATTCTTCTGTT
36301 TAGATTTGCAAGTGCTTTACCACAGTTACTGCATATAGTTCTTACAGAAAGAAGGAAGCT
36361 ATCAGTTGAGTTGTCTGCTCTGTCTCTTTCAATATATATTTTAAAAAGTCTCTACATATA
36421 AACATTTTAAGTATTATACTAAGTTAACATAATTTTCTTTAGAGTACTTCTTTAATGATG
36481 GCTGCCTCTTAAAGAGGCAGGAATCATTTCTGTAGTTTGAGACTTTTTAGCTAATTTATC
36541 TGTGATATGTAGTGGAGTCCCAGTTGAGGTAAAGCCGGGGCATCTTGTTTATTAAGCAGT
36601 AAAAGAGTCAACTTTTTTTTTAATTTATTTATTTTAATTAGAGGCTAATTACAATATTGT
36661 ATTGGTTTTGCCATACATCAGCATGAATCCGCCACGGGTGTACACAGAAGAAGTAGGATC
36721 TAGGGTAGTTAGGATACACTGAAGTAGACACCAGCGCTTTTTTTGGTTTTATTTTCCCTG
36781 TTGCTGTGCCACGTCTTTGGTATCGGGAAAAAACTTCAGTTGATGTCTTTCATTTTTCAC
36841 ATCATTCCGCCCCCCTACCCCCCACACACCCTCTGGGGTTTTTGCTTCCCATTACTATCC
36901 TGCGTATTACAGGATGTCTCCATAGTGTCGAACTTGAATGTAGAAATAGAGCATCACAGA
36961 GAGATGTTTCCCCCATTTCTGCTTCTACCCTAGGCAAGAGAGTCTCCAGGATTCTTCTCT
37021 GGACAAAGAGAGTGGGTCCAGAGAAAAGACCATGAGACACTATTATCTGGGGGCCCCAAG

Appendix B. Continued

37081 AAAAGGTTGACTTTTACCCAGTGATCTGATGGAGACGCCTCTGTTAGACAGCTCCCACCT
37141 GCATCCACGAAATTTCCAGTCTCATTCCTGTGTAGTTAATGATCATCGGAGACCAACACA
37201 AGCAGTAGGACCAAAGAGACTGAGGACACACATGCAGGTGGCAGGAGAAAATTTAAAACA
37261 TAATTGCTGTCCTTGGGAGATGGCAGAAGAGACCCTACATGTAGCACAAGTAGAAGACGC
37321 TATAAAAAAGAAATATTCTAAGAAGAATAGCCATACCTTGGAAACTAAAAAATGGTAACT
37381 GATATATAAATATAAATAGGAATGTCGAAAGCTAAGTTAGAAAATTTGTCTTAGAAATTA
37441 AAAAGAGAGAGAGATGGAAAAAGGGAAAAAAAGAAATAAGGGAATTGTGCTTTGAAAGTA
37501 GCGGTTGAGAAAGCACAACTTGGTCCTATTCAGATAGGATTTTTAGTCTATATTGTTGAT
37561 TCTGCCCTTGTTTTTTAAAATAGTAATAGAGAATAAATTTCTGCAAAGAAAAATTAGAAC
37621 AAAATAGAATAAATCATTAAATTACTACAACAAAGATGTTTTTTCATACTTCCTACTTAG
37681 CAATCACTCTTTGCTGTCATTTCATATCATTCAACAATATGATGCTTAAGTTCATTCCTA
37741 AAAAAGTAATGTATTATTTTTCCCTTTACACAAATAACTTAGGTTTTTAACTTACTTTGT
37801 CATCATGATAGCATAAACTGGTTTTGTTACTTAGACATAAGAACAGGGATTCAGCCAGTT
37861 TGCATTGGGGTAGTGTTGTCAGACTCTCTATTTCTCTGCTCCTTTTTGTGGAAACATGGA
37921 TTAATATTGAGATTTGCTCTAAATTAGCAAACTATCTGGTTTATCAGTTATTGCAGAATC
37981 CCACTGTTGAGATTAGAAAGCAGTCTGTCATTATGGCTTATTCTGTGCAAACTCTTTCCT
38041 CAAGATTGTTGGAGGAAGGCTTTTAATTTTAACCATTGTTGTTTTCCAG***ATAAAGCTCTG***
38101 ***ATG******GCACCAAATCTTGACTCCTTTGGACGAGATCGGGCACTGTATCAAGAACATGCCAAA***
38161 ***CGTCGGATTGCAGAACGGGAGGCCAGGAG***GTAATCTCCAGATGCTTGCTGTTGGTCGGAT
38221 TGTGAAGGGAGGAGTCTCCTTTACCTGGTTTGGAAACTCACTGCTTGTTATTTAG***GACTC***
38281 ***GTCGTAGACAAGCCAGAGAGCAGACTGGTAAGATGGCAGATCACCTTGAAGGCCTTTCCA***
38341 ***GTGATGATGAAGAAACTTCTACGGATATCACAAATTTCAATCTGGAAAAAG***GTTAGACTT
38401 TTATTTGGAAGTGAAATTCATTGTCATTGTGCTTTCTGCTTGGCTGTAGTTGTTACCCTG
38461 AAATAATGTGAGGTTTTGTGGCCTTTGCTTTAGAACTTGGCACTTGTTGCTTGCTTGCAC
38521 CTGTTTTTACCTGTTTTTATTACTGAATAGCGTGATGAATTCTCGCAATTAGTGGTAACA
38581 CTTTGTTGTGGGGTTTACTTAGTTACTTGCATTATAAATAATATTACTCTTCAGAATCGT
38641 AGAATTTTAAAAGTGACAGGAGAATGTTTCAGATCCCAAGGGCCACCCTAATGCAGCCTG
38701 TCTGGTCTAGAAAGGTAAAGGTTGGCAGTAGGGAGCCCTTCTCAGCCCCAAAGGGAGTTC
38761 CTGATTTGGGGAGTCCTGAATCATTCAACCACGGGGAATAGTTCGCACTGTTACATGAAG
38821 GCGTTTCCAGAAGGAACCCCAAGTAAAAGTCTTTATTTCCCACCATTGGCTGCAGTTATC
38881 TGTCATTTATTAACTTTGGAACATAGTTGGAGAAGCTTATTTTGTTTCTCCCGCTTTTTT
38941 ATAGTGAACATTTCTAAACATACAGGAAATTGGAAAGAATAGCACAGTGATCACTGTCTA
39001 CCTGTCACCCAAATCCAGCAGTTCTTTCCATCAGTTGGCCAAGTTTGTTTCGGATATGTG
39061 TGTTTCGCTGAACCATTTGAAATTAACTTACAGACATCATGGCACTTGCCTCTAAAAACT
39121 TTAACATATTTAATGTCTCCCCCCTGAAAAAGGATGCTTTTCTACCTTATTGTAACACTA
39181 TTACCACCCTTGAGAAAAATTAACTGAAACTTCTTAGTATCATATCTGTATTCAGTACTT
39241 CCCAAATTCTCAGGACTGTTTTTGTGGGGTGCTCTGTGTTTTCCAAACCTGGATCCACAC
39301 AAGGCTTCTGCATTGCACTTGATCATTGCACCTTTTTAATCTAGAATTAATGAATATCAT
39361 GAGTACCTTATAATACACACACTCCTACTATTTGTTCAAGGCATTTGCTTTCTGAAGGCC
39421 ATTTGTCTTATAAAATGTCCTACATTCTAAATTTGTTTCCCCGTGGTATTAACTTGTTCC
39481 TCCAGTCACTGTATTTCTTGTAAACTGGAATTTAAGTCTGGAGTCTTAATTAGATTCAGG
39541 TTAAACCTTTTTGGTGAGAATACTTCATACAAGATGCTCTTTCTTAATATATAATAGTAG
39601 ATGGCAAATGTTAGGCTGCCTTCTATCAGTAGTGTTTAGGTGGATCTTAAGAAGTAGATT
39661 GTCATGACTAGGTCTCTTCATTATAAAGAGTCCACTGTTCAGAGTCATATAGAGCATCAA
39721 ATAACTTCACTCAAAGATCCTAGCATCCATTGCAGATTCTTATCTGTTAGTTATTGTAGT
39781 CACCAAGCAACTTTGGAAGAACAGATATGATTGGTTATTGATGATCACGCACATCTGTTA
39841 TTTAAATGGTGATCTGTGGGCTGAAGAGCTGGATACAGAGTTTGTATTTTATGCAATTAC
39901 TCACACTTACTGTTCCATGGTAACTGAAATTTGAACTGAGTTTTGGGGAACGGGTGTTAT
39961 TTAGACTATGGTAGCAGATAATGGTACATATTAGAACTATGCAGAATGGCACCTGCCTGC
40021 ACTGTAGTTTGTGTGATAGACATTTGGAGGCTAAGTTTATCTCTGTTTCCTTTCCTTTAA
40081 GTTTCAGTTGATAATTCATTTCAGTCGCTCAGTCTTGTCCAACTTTTTGTGACCCCATGG
40141 ACCGTTCCTTTGTTAATGAGCCTATCTTATTACTTACTCAGGCTACAGAGAAAATGCTGA

Appendix B. Continued

40201 AAATAATTGAACTGCGTTTTTTCTACTGCATACTAAGTTAACATTTCTCTGTGGGGTTTC
40261 TTTGTTTTTGTTGGTTTTAG***ATCGCATTTCAAAAGAGTCTAGCAAAGTTTTTGAAGATGT***
40321 ***CCTTGAAAGTTTCTATTCAATCGATTGTATTAAATCCCAGTTTGAAGCCTGGCGTTCAAA***
40381 ***ATACTACACATCCTATAAGCATGCCTACATCGGCCTTTGTTTGCCAAAGCTGCTCAACCC***
40441 ***CCTCATAAGGCTGCAGCTTCTCACCTGGACTCCGCTTGAG***GTGAGTGTCTTCTCTCACTG
40501 TTGGAAAGCAGGATACCATGATGCTTGGGGAAGAGATGAAGCTTGAATAAAGTATTTTTC
40561 CCAGGAGGTACCTCATGCTCAGGGCTATAAGTGATACAGAGTATCACTGGGTGTAATCTT
40621 ACCAATCTTTCCTTTTATGTTCTGTCCCTTTTTAAGAGCACCATCTATTGCTTCTCAATT
40681 TGTATACAGTATCTTTCTCAACTAAAATTACAAGTGTTGACATGGAAATAGGAACATGAA
40741 TGAATAAGAACTTGGTCCTTTGATCCCAGAAAACTGTAGAACGTCATGGGAAAGCTCAGT
40801 GAAGAAGATGTGAAGTGGGGCCCTAAACTAAGTCAGTAGTGATGGAAATCAGGAGGATGA
40861 GATTTAAGAGGTGCAATCAGTAGAAACTGTTTGCCTGTTACCTAAGGGAGGTAATTGTCT
40921 AGGTAATTGTTGAATTGTCTATGTACATCTAGTGGTCATGTTTTAGATAACAGTTTTGTA
40981 AGTTACCAACATACGGGTGGAGCCGTGGGCAGGAATGAGATGGGCTGGGTTGAGCATGTA
41041 AACTATACCAAAAAGAAGGCTGAGGTGGAAGCCCTGGAAACAACAGGAGAAAAACATGAG
41101 CTCTGGGGACTCTTGGGCTCCTGGCTCCTGCTTGCCCAGCCCCCGTGGCGCTGCGCGCTA
41161 TGCTGTGTGTCCCAGTGCACAGCAGAGGCTCACGGCGCAGTGGTGCGCCCCTGCAAGCTC
41221 TCCATCTTCACAGCTCCTCCCTCTGTCCCGCAGCCGCCAGCTGTCTCAGCCTCCCTGAGC
41281 TGCCTTTCTGGCTCCTGACTCAGCAGCACTGCTCTGCTTGGGGGTTCCCTACCCTGCTCT
41341 GCTCTCCTGACCAGGAGTCACGTCCTTTGACTGCCTTCTTTCAGGGCCCATAGCCCTATG
41401 CTGCCTCTGCTGGTCCATTGTCTAAGAATAGATGTTTCCTATATTTTGTCACATATTTAG
41461 TTTATGGCTGAAGGCTGAATCCAGTTCCAGTTACTCTCTTTGTGGCTAGAAGCAGAACTA
41521 GCCACTGTTTTAAATGTTTGGGTAACCTTTTAAATATTTTCTAG***GCAAAATGTCGTGACT***
41581 ***TTGAGAACATGCTGTGGTTTGAATCTTTGCTGTTTTATGGTTGTGAAGAGCGAGAGCAAG***
41641 ***AAAAGGACGATGTCGATGTCGCACTGTTGCCTACCATTGTTGAAAAGGTGATTCTTCCTA***
41701 ***AACTAACAG***GTATAGATTACGGAATTGAACATTCACACGCATTGGGTTTTTCATATCTAT
41761 TGTTAGGAGTTTTTAATCAGAGCATATATTCTCATAGAAATTACATTATTAATAATGAAG
41821 TTACAGTCAGACCCAGAGATGGCTAAAGGGTACCTGTCTCTTACCTTGGGACTTTGAAGA
41881 CCACCTTGGGGAGGGCCTGGAATTGGGGGCAGTAGGGAGATTTTAGACTCTTAAGGACTG
41941 CCTGAAGCAAAACTGAATACATAGTTTTGGGAACAATATGCATTTTTCCAGGGAGCAGTT
42001 TCATTTCTTTCATCAATTTCTCACAAAAGTTTAGAAATCATTGACCTAGGGAGCTGTTTA
42061 AAATATGAAAAAAGTTTAGGTTCAGTTAGAGATTCTTGACACTGGGACAGAATCTCCTGG
42121 GATATAAAAATCCCCAATCTTTATTTTCACCAAGAAGCAGAAAATAATAGGAATCTGTGT
42181 CTCCTCTTCACCTTTGCCTAATTGTCAACATTTCTGTGGTTAATTGGACAAAGACAACTG
42241 AAGCTCATGCTCTAGAGGAAGAGTCACGATTGGCATTTCTAAACGGAGAACTGCTTGACA
42301 TCAGAGTTACATCAGCTGCAGTTTACGTCTATATCTCAGTACCTTCTACCTTTTTTTTTC
42361 CTTAATAG***TGATTGCTGAAAATATGTGGGACCCCTTTTCTACAACACAGACTTCAAGAAT***
42421 ***GGTTGGAATTACTCTAAAATTAATAAATGGATATCCTTCAGTGGTGAATGCAGAAAATAA***
42481 ***AAATACACAG***GTAATTTAGTTATTATTTATGAAAAGTTTAAAAATTTTTATGTATCTTCT
42541 CTCCCTTTGAAAACGAGCAAGTTGAGAAAACGAGTGGGTTATAGTCAGAAAATCTGATTC
42601 TCCCACTTGAGGTATCTGGTCCTTTATATAGAAAATCTGAGATAGTGGTTTTTAATTTTA
42661 AAAGAGTCTAGATAGCTTCTTATCTATACTGTTTTCATCCTTCTCACCGCCCCATCCCCT
42721 TTCACAATCAGACATCCCTTAAAGAACTTAATTTTGGGGGAGGGAAAGAAAAGCAGAAAA
42781 GTACTGGGATGTGTACCAATTGATAATTTAAATCCCGAAACATGTAATAGCATGTAAAAC
42841 AATCAGGTGTTGTTATTGCATAATTAGTTTCCAAACTTTAAATTGCTGCTGCCCCCACTG
42901 AACTTTCTTACAAGCAATATAGACATAGGAAAGTACTCTTTATCATTGTTTTACTTTCTG
42961 ACCTCATTTTGAGAAGAACCAGACTTACAGGTCTGATTTTAAACCTTTATATAGTAGATT
43021 AAGGGGCTGGAAGATTTCTAAATGACTTTATTACTTGGTAATTGATGAGGCGTGAACTAA
43081 GAAGAAAGACCTTTGGTGAGAAAGTAAATTTGAGCTGTTCACTCAAAATTGAATACTCAA
43141 TTAGGTGCTTTGGAATGTGGCTTAGTTTTATTTCCTAAACTTTTACCATAACCCTTATTA
43201 TAATGCATGATCTATTTTTAACTCTTCAG***GTATACCTAAAAGCACTTCTATTGAGAATGA***
43261 ***GGAGAACTTTAGATGATGATGTATTCATGCCCTTGTATCCCAAAAA***GTAAGTTATTCCTA

Appendix B. Continued

43321 AAAATGTTAAAGATGATAATATTCTCTATTGTTTTTCAGAGAAAGCTTCTTAAATTTTGG
43381 GAAAACTAAGCGTAGCTTGTAATGTAAACTTCACCTAAGTAGTCTAAACGTGTAGCCCAG
43441 ATTGAGGAGCTCATTGTGGAAACCGCTTCCCTTAAGCAGAATAAAACAAACAGCGCTCCG
43501 TTATCTCAGAATACATAGCCATTACCTTTTAAAATTACTGTCTTCTGGAATACAATTGAA
43561 AATCAGAAAAATTCATTGCTTCCTTTGTTCACCTCTTACTTAACAGAAATCTTCTAGAAA
43621 TAAGATGGAACTTAACGCACAACTTTTGATCATGATGTGTTTCTCTTTTTTTCCTTAG***TG***
43681 ***TCTTGGAAAATAAAAATTCTGGGCCTTACTTGTTTTTTCAACGACAGTTTTGGTCTTCAG***
43741 ***TTAAG***GTAAGTGTCTACAAGCTATCTGAAAGCTTTGGATTTTGTTTGACTTTCATACAGT
43801 ATATTTCCAAAAAGTATTTTAATATTTGGTTAAGTAACTTCATAGGTTTGATGTTTCCAG
43861 AGGAGAAAATGAATTCTGAGTCCCAATAAGGTCCAGACTACCCAACAAAATGCCTTACTA
43921 AACCACAGTTGATTTATTCAGCAAATAGAGTACCAGGTACTCTTCTAAGTTGGGGGAATA
43981 TAGCACTGAATAAGATAGAAGTAGAGCCATCATGGTGTTTATAACCTAGCACAATTGACT
44041 AGAGCAAATAAGTAACCTCAGGTAGGAGGAGTTTTGAAAAAAGAAATTCAGAGTTGTATT
44101 GGAGCATATGACAAGGGGCATAGAAGATTCCCATCCATCCTGAGAAGTGATATTTAGGTT
44161 GTTAGTTGGCCCTGAGGTGAGGGAGAGAAAGATATACTAGAACTGAGAGAAGTCCAGGGT
44221 GGCTAGAGTTAGGGAAGAGATAAGCAATCCAGATGATACAGAAGCTATAATTTACGTGGA
44281 AACATTATAGGAGACTCCTCTAATCAAGATTATAACCCAAATCCCCCAATTCCTAAGACC
44341 TTTTTATTGTCTTATAGTTTTCTTTGTAAGAATGTCTTAGGAATGGAATTATATACTAGT
44401 TAGCCTTTTGAATCTGGGTGCATTCATGTAACATAATGCATTCATGATTTAGTGTTATTT
44461 TATGTTCATTCCTGTCTTCAAATGGTGAATATAGCATTTAAAAATTTTTTATTGAAATAT
44521 AGTTGACTTGACAGTGTTGTATAGTAGAGTGATTCAGTTATACTGAATCATATCCTTTCT
44581 CAGATTCTTCTGGATATACCATTTTTTGGTGCATTTTTTTATCCATTAGATAGTTTACAG
44641 TTTGGAGTAATTATGAATAAACCTGTCAGCATACAGGTTTTTCTGGGTATATAAGTTTTT
44701 ATTTCTGTTTGATAAATATCTAGATTATTAGGTAAATATCTGAAAGCTGTTTTCTAAACC
44761 AACAGTATTAGTGTTCTATTTCTTTCTCACACACCTCAGTATCTTTCAGGGTTTTTATTT
44821 TTTAAGCCATTCTCCAAGTGTATGTTCAGGTCTTGTTAAGGTTTGTGTTTCCCTAATGAC
44881 TGATGATGCTGAGCATGTTTTCATGTGCTTACTTACCATCTGTATGTGGCTCAGATGGTA
44941 AAGAAACTGGCTGCAGTGCAGGAGATCCAGGTTTGATCCCTGGGTCAGGAAGATCCTCTG
45001 GAGAAGGGCATAGCAACCCACTCCAGTATTCTTGCCTGGAATACTCCAAGGACAGAGGAG
45061 CCTGGTGGGTGACAACCCATGGGGTTGCAAAGAGTTGGACACAACTGAGCAACTAACACT
45121 CATGTTCTTTAGTGAAGTATCTTTGCAAATCTTTGGCCCATTTTTAAAAATTGGGTTTTC
45181 TTACTATTGAGCTTTGAGTTTTGTATTCTTGATACAAATACTTCACATACATGATTTGCA
45241 AACTTTTTCCCCTGAGTCTGTAGCTTGTCTTCATTCTCTCAGAAGTTCTTCATTCTGATG
45301 AAGTCCAGTTACTACTTTATCCTGTTATGGATTGTGCTTTACATGTCACATCTGAAAAAT
45361 CTTTGTGTAACACAAGGCCACTAAGTTTTTCTCTAGCCTTTCTTCTAGATATTTCATGGC
45421 TAAGCTCTACATTTAAGTCCGTGATCCATTTTGAGTTAATCTTTATATATGGCATGAGGC
45481 CTGGTTGTTCAGGTTCATTTGCCCCAGCACACTTTGTTGAAAAGATGATCTTTTATTAAA
45541 TTCATTTGCTTTTGAAAATCATCTGCCCTTATATGGGTCGTGGAAGGGTTTTTAAGTAGG
45601 GAGGTGACTTGCAAAATATTGCCATTTTTTAGATATTGCAGTTGCTGTGTGGAGATAATG
45661 GTTTGAAGTAGGTAAAGATGGTAATAATGAAAATGGGAATTAGTACTTACCACATATTTA
45721 CTGTGTTCCAAGCGCTGTACCACACTCTTTATTCAGATTATATTCTCTCGAATCCTCACA
45781 ACAGTCCTGTGAGCTTAACTCCACTGATGGGACAGTGGGGTGCGGAATCACCTACCCAGG
45841 CTTACTCCTTTGTTAACTGGTAGAGATGGGAGTCTCCTTCAGGCCAGTCTGACTCTTCAG
45901 CAGTTATTCTCAACTGCGGTTCTGGGAACAAACACTTAGGTCCTGGTGCCCTCAGGCCTC
45961 TATCGTGTATGTATCTGGAATGGTATTAGTTTGTCCCATCCTTGGGAAAACTAAAGAGTT
46021 ACGCAGATCATTTTCTGTAGGATTGAGTTCTGTCACAGACCCTGCTTGAGAAGAGCTGTC
46081 CCTGAGCCATAAAGAGATGACGGCAGCCTCCGTTAGGGGCATGGCAGCGGGGACCGAGAG
46141 AAGGGACAGCAGGGGTGGGGGCGGGGTGAGACAGCTTGAGGGAGAGGCTGGGAGTGACAT
46201 GTCGGCTGTGGCCTGCTTGATTTGTTGGATAATGTTGTATTTCCTAGGATGAGAGATACT
46261 GAGAACATAGAAGTATTTATGGTAGAATGCGTGGGGACTTTTGGGTATTGAGCTTAAGTA
46321 GCTTCCAGTGTCCTGGTTCATGAGGTCTGGTAAACATGCAAGTCTTACAGGTTAGTCTCT
46381 CAGTGGTGTCTGATTCTGCGACCCCATGGACTGTACCCTGCCAGGCTCCTCTGTCCATGG

Appendix B. Continued

46441 GATTTTCCAGGCAAGAATACTGGAGTGGGTTTCCATTTCCTTCTCCAGGGGATTTTCCTA
46501 ATCCAGGGATTGAACCCAAGTCTCCCGCATTACAGGTGGATTCTTTGCCAATGAGCCACC
46561 AGGGAAGCATGCAATATGGTAGTCGGAAAGAAAATTTCCATCTTTTGGCATAGTCATAGG
46621 ACTATGACGTTTTTCATGATTTTCAAGGGCGGACATTTATCTAAGAATAATCACTTCTCT
46681 GCCAGTACTTGTTATGATGGAAACTGTTCTTACCTGCATTGTCCACTACAGTAACTACTA
46741 ACTCTGTGTGGCTATTTAAAGTTTAGTACATCTCAGTCATTCTAGGAAATATTCAGGCCC
46801 TTCAAAACTGCTTCTTAGTGCTTGGGCAAGACATGGTCCATGGTATTTCATAAGGTGTTT
46861 TAGTGTGTAAGTAATTTACTTTGTATGTACTTAATTTAATCGCTTTACATTGCCTCTCAG
46921 TACTTTTCTTCCCTTCTCAAAAAAACAAACTTGTAGTAACAGAGATCTGATTTGTGGTTG
46981 CAACAGGTGGGGTTCAGTTCATTTCATTTAGTCGAGTCCGACTCTTTGCGACCCCATGGA
47041 CTGCAGCACACCAGGCCTCCCTGTCCATCACCAACTCCTGGAGTTTAACTCAAACTCATG
47101 TCCATTGAGTCGGTGATGCCATTCAACCATCTTATCTCTGTTGTCCCCTTCTTCTGCCGT
47161 CAATCTTTCCCAGCATCAGGGTCTTTTCCAATGAGTCAGCTGTTTGCATCAGGTGGCCAA
47221 AGTATTGGAGCTTCAGCATCAGTTCCTCCACTGAATATTCAGGACTGATTTCCTTTAGGA
47281 TGGACTGGTTGGATCTCCTTGCAGTCCAAGGGATTCTCAAGAGTCTTCTTCAACACCACA
47341 GTTCAAAAGCATCAATTCTTTGGTGCTCAGCTTTCTTTTTAGTCCAACTCTCACATCCAT
47401 ACATGACTGCTGGAAAAACCATAGCCTTGACTAGATGGGCCTTTGTTGGCAAAGTAATGT
47461 CTTTGCTTTTTAATATGCTGTCTAAATGGGGAGATGGGGTAAAGTAATCAAAAGATACAA
47521 ACATCCAGGATGTAAAGTTCAACATGATGACTGTAATTAACTATAGTGTACTGTGTATTT
47581 GAAAATTGCTAAGAGAATAGAAAAAAGATGTGTAACTCTGTGAAGAGAGGGATGTTAACT
47641 AAACTTGTGGTAATCATTTCACAATATATATTTTAACTATCACTATATGTTTAAATTGTT
47701 AAACTTGTGCAATGTTACATGTCAATTATATCTCACTGGAACTGGGAAAAATAATTTTTC
47761 TCTTCTTTCTAG***CTCTTAGGGAATTTTCTTCAATGGTATGGCATTTTCTCAAACAAAACT***
47821 ***CTTCAGGAATTATCAATAGACGGTTTATTAAATAGATACATTCTCATGGCTTTTCAGAAT***
47881 ***TCAGAATATGGAGATGACAGCATCAAAAAAGCCCAAAAT***GTGAGTTAAATGACACATATT
47941 GTAGTTTTTAAAGCATATACGTTAAGATGAATTGTTTTGTTTGAAGAGATTTGAGGAATT
48001 TATGAAAACCCCTTTGATTATTGAATTTGCTGTAATGTTGACAGTCAGCTTGCCGCCCAG
48061 GTGGTCCCAAATCCCCTTAGCATTTTTTATGTAAAAATTTGTGCTGTTGTATGTCTATGT
48121 TTATCCTTCTATCCATCTATCTATATCAATATTTATTTTTGTAGGTACTAATAAAAGTTT
48181 TAACTTGGAGATAGTTTTATAACAGTTGAATGAATACTATTCTAAGTGTTACTAAAGAAA
48241 TGGTTTCTTTTCTCATTTAAGAAGTAATTTTAGTTAATTTTGTTTTACTATACATGAGTT
48301 TACTTTATATGCATGTGTTATATACACAATTTTGATAGTTTTTTAATTTTTAAACAATAA
48361 TACAATTAAATTCTGCAAGTACAGTATAGCTTTTTGACCTGTACCATGTCAGAGTAATTC
48421 TCTGACTTACTCCCTCACCATCAAATATTTTTGTGTCTTGTTTCCTGTAAACATAGATTT
48481 TGTTGTATATTACAGTATAAACATCAACATTAGGAAATTACATTTATATATTGTCACCTA
48541 ATTCTTAGGGCTCTTTTCAAGTTTTATCAGCTGTCTCAATAATATCCAAAGAAATTGGCA
48601 AAAGAAATTTCCAGATCATCCTTTGCATTCAGTTGTCATTTGAAGGTTTCCTTGACCTTC
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48721 TTTTTCTGATATTCTCTCATGGCTATATTCACATTTTGCATCTTTGGCAAGAATTGTACT
48781 AGACTTGATTAGTAGGGGCTCCTTCAGGTTGACCTCTGTGACCTTGTTTCTAATTTGGGG
48841 GAGCATTTCTTTGTTTCCTGGCACGATGTTCCAGGCACACCTGGTACTTGCTTGGCCCAG
48901 TTCGAATCCGTTGTTTCTGCAAGCATCCCCAGGTTCTTCGGTGGGGCGCCTTGTCTGCCC
48961 ACTGGCCGCTTTCTCGCAAGTGGGAACAGGCTTCCCCTTGCTCAGGTTTCGGTTCCCCAT
49021 GCCGAGTTGCTGCCATGCCGTGCTGGCCCTGCTTAGGTGCCTGCCCTAGGCTGCCCCTCT
49081 GGCATGATGCTCTCTCCACCCTGTGCTGGGCTGGTGTGGCTCCCGCTGTCGGCATGGATG
49141 CCTGCTGACTGAACGGTTCTGCGGGGGAGGGGCTGCGATTTGGTGTTTATGCTATACATA
49201 TTATTACATCAAAAACTGCTGACAAAAGGTCAGCTCAATTCATGTAGGGCTTGGAAGACT
49261 GGAAAGTGAGGCAGTTAGTTATCAATTTTATGTCCCCAATGTACTTAAATTTTGGAGAAA
49321 TTATCTTTTACTTTGGGTGTCTGTCAAAGTTTAAAATTAGAGTATATCCAAGTATATTTG
49381 TATAGCTTATGTAGAAAATCTGGTTTAAAAGAATATCCCTTCTAAGTGATTAGTTAATAC
49441 ATAGCTCAATAGCATATCTTCCAGTTAAATACATTATACCTGTTGTCTAGACTTGGCTTT
49501 TAGACACTTTATTTTTGTTTTAACTGTTCTACATCTTTCTTTTCCATAG***GTAATTAATTG***

Appendix B. Continued

49561 ***TTTCCCCAAGCAGTGGTTCGTGAATCTTAAAGGAGAAAGAACTATTTCTCAGTTAGAAAA***
49621 ***CTTCTGTCGATACCTTGTACACTTAGCAGATACAATTTACAGAAACAGTATTGGGTGCTC***
49681 ***TGATGTGGAAAAAAGAAATGCAAG***GTAATTATTTATAATTACTGAATGAAACAGTGATCT
49741 AGAGTCTTAGTTGTTGGTCTAGTATTATTAAAATGCTTTTGGTAGAGACATGTTTATATT
49801 TTACTTTAAATTGTTCATTGACTTCATATTGATAAACCTGCCAAAAATGTGCATTGTATT
49861 TTTCACTTTTGCAACTCTTACTTAATCTTTTTTCAGTATGTTATTTATTCATGAAAATAT
49921 TAAGGGAAGACTTACTTCTAGTAAGCACCAGCTGTAAGGGGAATGAATGAATCATTATTT
49981 TCTCTGCCTTCCCCCATGAAAAATTGAGGAGACTGTAGACTCTTAGAATGTCATGCATCT
50041 TGGTCTTTCGAGGTTCATTTTACAGATATAAACCCAAGAAAAAATAGCCACTGAAACCCC
50101 AAGTATATTGAAGGTTATATCCCAATTTGGGGATGTTTGTGTTCTATGTTAAGTTTTATA
50161 TCATTTTGGTGTTGGTTGCAGTGTTTTGGGGAGTTTAAATATAAACAAACAGTATTCACT
50221 TTTCTACAGAAAAGAATTTAGCAACTATTTAAATACCTGCTGTGTGCAGTGCACATAAAG
50281 TAGGCTCCATGTGGGTCACAACATCTTACAAATTATAATCCTTCCCAGAAAGGTGCTTAC
50341 TACAGTTCTATAGAGAAGAGACAACTGACAATGTCACACTGACTCAAACTAATGTTAAAC
50401 GTACTTCCTGGACCAGCAGCAGCAGCATCACCTGGGAGCTTTGTTAGAGACACAGATTCT
50461 CAGGCCTCACCCCAGACCTGCTGAACAGAATTCCGAGGGAGGGGCCTGGTGGTCTGTGTT
50521 TCAGCACTCCTCTAGCTAATTCTGATGTATGCTAAAATTTGAGGATTGCTGAGAATGACT
50581 TGTAGCTAGGTGATAAAGGAAGCCAGATTATACTTTATTATTGGATTTTAGAGAAATGGA
50641 AGTTATGCTGGATCTTGATAGATTGGTGATGAATACTCACAAATACAATTTGTGTTCCTG
50701 GTATATTCTGGAAGAGCTAGGACTTGGGTTTGGAAGGAATGGGTGGCTGGCCTCTGAAGT
50761 AGTTAGGCAATAGATTGCTTTCGTTCCTTTCTAGATATAAATGAAAATCATGGATCTGAA
50821 CAAGATGTGGGGTGTTTTGTTTTTTTTAAATGAGTCCTTTTTCTGGCTACTCATCTAAAC
50881 AGGAAAGTTTTTTCTTTTTTAATATGGTTAATTTTTTTCACATGCTATAACTTGCTTATC
50941 TCTGTTTCCTTTTTTCTTTTTTAG***AGAAAACATAAAACAGATAGTAAAACTCCTTGCAAG***
51001 ***TGTTCGAGCTCTGGATCATGCTATGTCTGTTGCAAGTGACCACAATGTGAAAGAATTTAA***
51061 ***GTCTTTGATCGAAGGAAAATAG***ATCTTTGAGACTAAAAAAAAGCCTATTTGCTCTAATAC
51121 CATTCCCAGTGTAAAAAATTTGTATATATGTAAAGTTTCTTAAACTGTAAAATATTGACT
51181 GATTTTAATACAAAGAATGTTATTAATATTCAATACTGTGTCCTTAAATGCAGTTTCTTC
51241 ACAATGAAAGGAATTCAATTTCTAGGAAGTCTATGAATGGTTTCCTTACTGCCTGCCATA
51301 CTTTGTTTACAGAAGTATTTTATATGGTCAATTAAATTAACCCTCTCAGTTAATTGTTCT
51361 CTGTAAAGGATGGGTGCAGTGTATATTGTATAGTTATGCATATGTATATTTATACCACCA
51421 TGGAGTTACTTTCACACTGGAAATTTGTGGTGTTTTTCTACAGAGCAAGCCTTTTAACAG
51481 CAGAAAAATTAGTCAGTAGCAAATTGTCTTAGGGAAATTACTACACTGTTGCAACAAAAG
51541 GGCTGGCAAGTGACTATTCATCACTTTTGGTTAGCAGTTGCTGTCACTGAAGCTGTGCTG
51601 CCTTAAAATTGAGAGTTGAGTGTGGACTTAGTCATTGGTGAATGGTGAACTTTTCTTCCT
51661 TGAGTTAGCATTACAGTCAACTGTTCGTTTAATGTGAGCTCTAAATAATATATGCAACAT
51721 AGGTTTCTGAAGAGAACACAGACATTTCATTTCTGGAAATAATAGACCCACAACCTAACT
51781 GCATGGTTTTGGGCTGACTGTCCTGTCTCATTCATGATTTTACTCCCTGACCAGCTTAAA
51841 TACATGCATTTGTATTTTAGTAGTCTAGCATTATATTTAAAATCCCACATATGTGAGTTA
51901 TATATGCAATAAAGAACACATTTTGCTGATTCCCCACACCCAGGCAGTTTAATATCCATG
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52021 CCCATGGACTGTAGCCTACCAGGCTCCTCTGTCCTTGGGGCTTCTCTAGGCAAAAATACT
52081 GGAGTGAATTGCCATGCCCTCCTCCAGGGGATCTTCCCAACCCAGGGATCAAACCCAGGT
52141 CTCCCACATTGCAGGCAGATTCTTTACTGACTGAACCACCAGGGAAGCCCTATAATATTT
52201 ATACCATGTTGTAAATGATTCTGTGATTTTCAAAAGAGGAACTTTTTGGAAATTAAGAAC
52261 TACTGTTGGATAGTTACATATGTAATTAGGCTTTAGCCAAGCATTAAATTAATTTTTAAA
52321 TAGAACCATTGTTGTATTTGGCTGGTGACTAATTTTAGTTAGAGCATTTCAAATGACTAA
52381 TATAGTGAGATCATTAGCATGAGTAGCCTTGGTCAAGTCAAATACTATTTAGAACAGTGT
52441 CATGCACTCTTTATTGCTCTTGTCAATTTTATCTTTATTGTAGGAACACTGCGTATCCCT
52501 ACAGATTCATTCTCAATAAATTTTGTTACAACTCAGTGGCCTACTAAATAGAACTAAAAT
52561 AGAAGGCTTATTCTCAACTTCAGTGTACTAAAATATATAAACATGTATATATGTATATGA
52621 CATGCACATCATTTGTGCCTTAAATTTGTTTTTCGAAACCTATTGGACTGATTACTTTTC

Appendix B. Continued

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52681   CTATATCTGCTTTTAAGGACTCGAAGTTAAACTCAGATTCTTCATCCTATAAAATCCATC
52741   AGGCTCTTATACTTTCAGGAATGAGTTTGGTATAAGGTGAATTTAGCATAGAACTGTGCG
52801   TTAAACAAGTAGTTCTCATATCTCAACCAGAAGTTTTTTATTCCTGAAATAGGTAAAGGA
52861   CATTCTTTCTTAGGAAGGAAATTGTCAAATGTTTAAAAGAGGGTGACATTTTAATAAAGC
52921   TTGAGGTTTTTGTCTTTTCAGTTTTTTCTTTTGGCGCAGTCTTCCAAAATACTTGCAAAA
52981   TAGATGAGGAGGGTTTTCGAAAAGGAAAGTTATTGAGATACTATAAACCACAGTCATAAA
53041   GACCGATAACTACTGTTGTGTACCAGATGATGCTGGTGAGCCACATCCTTATTGATGTCA
53101   TGGTGCAAACACCTGCAGCTCTGCCGGAGGCCTTTCTCAGCCTGTGCTACAGGCTAGAGG
53161   GTCCACCACCTCTGGGAGCAACCCTTGGCCAAAGAAAGGAGGGGGAGGGTAAGTCCCCCA
53221   GTGTGTTAGTTCAGGTCCTCTGGGGAGCAGTCCCTGAGATGGCATTGGATATACAAGAGA
53281   TTTGGGGGGTGGAAATGATGATGGGAATGAAGGAGAAGGGAGCGGGAGGAGGCTGAGAGA
53341   GCTTTAGAGCCAAGAGATTGTGAGCAAGTGTGACCTCTGAGGGTGAGTGGGAAGGAATTA
53401   AGGAAGGTGTCAGACATCAGTGGAGTCCTCAGGAAGTTTGAGCAAGGCTGACGGATTCCT
53461   CAGTACAAAGTCATGGTCCTGAGGAGTTTTGTGTTTCCAGGAGCTGGCCTGCCTTTGTGT
53521   CTCTACCAGCTCAGTAAGGGGTAGAGGACAGTTCTTGAGGAGCACAGCCACTCTTTGGAG
53581   ATGTAAAGGAGCATGCTTTCACGTTGAACATACCAGCTTCCTAGTTTCAGGAAAACAGGT
53641   ATGATCCGTGTCATCTCTCACAAGGCTGCAGTTGACTTGAACCAGAGTTGTCTGCAGTGA
53701   TGACCTCATCACCCAGAACTGGCCCAGAATTTTCAGGAATCTTAAGTGACTTCCTCATGA
53761   GAATATACTAAAATACAGAGTTCCCTGGTTGTTTTAAAGATAGGTTTGTGGGGTACGATT
53821   TATTATAAATGTTTTACCCTCTATAAACACATATAGTTCAGGGCATTTGGACAAATGCCT
53881   AATTTTGTTTTAAATGAGAATTTCCCTTGAAATTATGACATCCTCCTAAAAATAGTCATA
53941   TGTTTTAGAGCTGCAAGATACAATATGAAAATGTCAGTTGCTACATTGTGTTTTTTTATG
54001   GTTACAATGACTCTTCTCATTTCTAACACTAAAATATCCATGTAGTAAAGGGAGTGTTGC
54061   CATTCCTTGATTATGACAAAGAACACTTTGTTGTTTCAAGTAAATGAAAATAAGAACTGT
54121   CTCTGGGCATTGGGTTCATAGTTTCTAAGAGACTGCAGATTGCTCAGCAAAGGCAGACAG
54181   GCTAAAAATTGTTACGTGGAATCAGTATTGATACACAATGTTGACTGGTCACTGGCCTGC
54241   TTCTGGACCTCTGAGCCCCAGTTATTGTACTGGCTTCCTCCCAACCCCATCACCCCTAAC
54301   TCTCACAGAGGCTCAGAGGTTAGACTCATCCAGTACGAGGAAAATGCCTAGCAAAGCGTT
54361   TCTCAGAATGTATACTGTGGTTCACCAGTTCAGGGGCTGTTACTT
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